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Contents

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Cellular Control of Renin Secretion

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Contents

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1 Introduction

The renin angiotensin aldosterone system (RAAS) is one of the basic regulatory mechanisms for controlling extracellular volume and blood pressure. The activity of the RAAS is mainly controlled by the rate of renin release into the circulation. The presence of renin has been demonstrated in a variety of tissues, including kidney, salivary glands, placenta, blood vessels, and various endocrine glands (Campbell 1987). Although renin obtained from these tissues, in particular from the submaxillary gland, has been important for the elucitation of the structure and intracellular processing of renin (Panthier et al. 1982; Cantazaro et al. 1983), so far only renal renin has proved important in the regulation of the RAAS. Therefore, unless otherwise indicated, only renal renin is considered in this review.

Within the kidney, renin is produced and stored, and then released from the so-called granular juxtaglomerular cells (Barajas 1979; Taugner et al. 1984). These cells are modified smooth muscle cells and are located in the tunica media of the afferent arteriole, adjacent to the glomerulus (Fig. 1). Morphologically they resemble vascular smooth muscle cells, but have with some typical

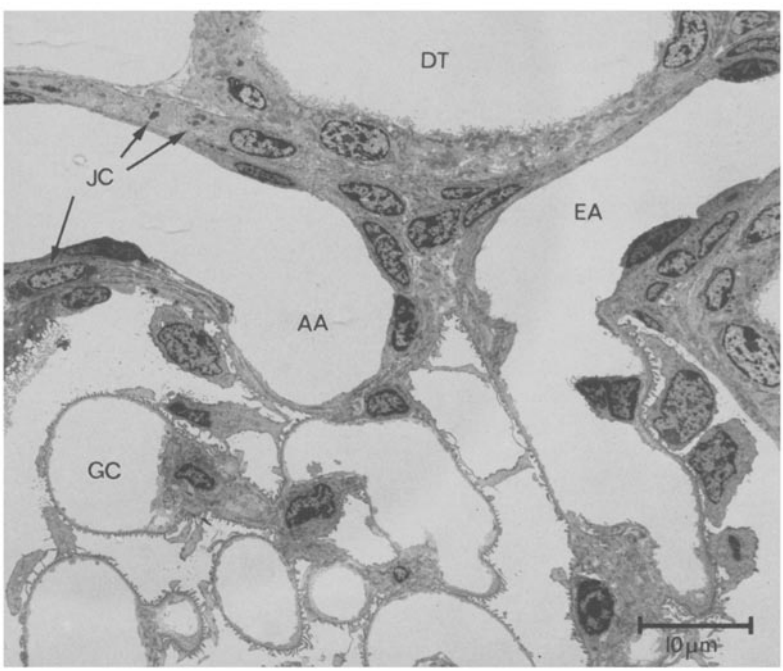

Fig. 1. Electron micrograph of a juxtaglomerular apparatus in mouse kidney. *JC*, juxtaglomerular cell; *DT*, distal tubule; *AA*, afferent arteriole; *EA*, efferent arteriole; *GC*, glomerular capillary. Courtesy of Maria Harlacher, Heidelberg

characteristics of secretory cells, namely a well-developed endoplasmatic reticulum, a prominent Golgi apparatus and membrane-bound granules containing renin (Barajas 1979; Taugner et al. 1984).

Since the rate of renin release from juxtaglomerular cells has a direct effect on blood pressure, and because an enhanced rate of renin release is known to be a major cause of hypertension, the mechanisms controlling renal renin release have attracted interest among physiologists, pharmacologists, and clinicians.

So far, four basic mechanisms for controlling renin release from the juxtaglomerular cells have been identified (Davis and Freeman 1976; Keeton and Campbell 1981). These are:

1. Intrarenal blood pressure
2. Influence exerted by the macula densa segment of the distal tubule
3. The sympathetic nervous system
4. Humoral factors

Despite a large body of findings on the modulation and alteration of renal renin release, the intracellular mechanisms by which renin release is controlled within juxtaglomerular cells are not clearly understood.

During the last decade the physiological and pharmacological mechanisms which alter renin release have been sufficiently reviewed (Davis and Freeman 1976; Keeton and Campbell 1981; Fray et al. 1983 a, b; Hackenthal et al. 1983; P.C. Churchill 1985; Vallotton 1987). This review therefore focuses on the intracellular processes that govern renin release from juxtaglomerular cells. The available information is discussed in relation to present knowledge of general mechanisms of secretion. It has been shown for a variety of secretory cells, including those of the adrenal and pituitary glands, exocrine and endocrine cells of the pancreas, neutrophils and platelets, that the secretory process is triggered by guanosine triphosphate – (GTP-)binding (G)-proteins (Burgoyne 1987), phosphoinositide turnover (Berridge 1987), intracellular levels of calcium (Penner and Neher 1988), and cyclic nucleotides (Rasmussen and Barrett 1984).

Evidence exists which indicates that the cytoskeleton (Hall 1982) and cellular electrical properties (Petersen and Maruyama 1984) have roles in the regulation of secretory processes. Therefore, special consideration is given in this review to the role of cytoskeleton, calcium, cyclic nucleotides, phosphoinositide turnover, G proteins, and the electrical properties of juxtaglomerular cells in the control of renin release. In this context, our own results, obtained from studies on isolated rat juxtaglomerular cells, are presented.

The effects of the four basic mechanisms which control renal renin release and how, these can be explained by present knowledge of the intracellular control of renin release, are also discussed in this review.

2 Renin Synthesis and Secretion

2.1 Experimental Models

As with many other basic physiological investigations, the study of the intracellular control of renin cannot be primarily performed in humans. Therefore, the results described in the following paragraphs have been obtained predominantly with rats, mice, and dogs. No essential differences in the control of renin release between rats, mice, dogs, and humans have been discovered so far. Therefore, it is assumed that the findings obtained with preparations from laboratory animals are also representative for humans.

The main reason for the absence of detailed knowledge about the cellular control of renin release is the lack of suitable experimental models. Obtaining insight into intracellular mechanisms within juxtaglomerular cells from studies using whole kidney preparations is difficult. The effect of a substance found to alter renin release in these preparations may result from a variety of causes, i.e., a direct action on juxtaglomerular cells, an influence on either the baroreceptor or macula densa receptor, an alteration in the activity of the intrarenal sympathetic nervous system, or altered sensitivity of the juxtaglomerular cells to certain hormones. To circumvent these problems experimental models have been devised which allow more direct access to juxtaglomerular cells. The hydronephrotic kidney model (Bührle et al. 1986a) is considered to be free from tubular influence, and experiments studying renin release from renal cortical slices are performed assuming that the baroreceptor and the macula densa are inactive in these preparations (Keeton and Campbell 1981). Other approaches to obtaining a more direct insight into processes within juxtaglomerular cells are the use of microdissected afferent arterioles (Itoh et al. 1985a, b), isolated glomeruli containing fractions of the afferent arteriole (Blendstrup et al. 1975), and the use of suspensions of isolated renal cortical cells (Lyons and Churchill 1975; Khayat et al. 1981). The disadvantage of all these preparations, however, is that the percentage of juxtaglomerular cells is low.

It is clear that hydronephrotic kidneys, renal cortical slices, isolated glomeruli, and renal cortical cell suspensions are not optimal for metabolic studies on juxtaglomerular cells, since the tissues used are obviously not fully representative. This disadvantage led to increased efforts to develop experimental models containing a higher percentage of juxtaglomerular cells. In 1982 Rightsel et al. succeeded in cloning juxtaglomerular cells from rat kidneys, but unfortunately, these cells had only a very low renin content. Moreover, the cloned juxtaglomerular cells did not develop the renin-containing secretory granules which are typical of juxtaglomerular cells in situ. Galen et al. (1984) suggested that cultured human tumoral juxtaglomerular cells could be a use-

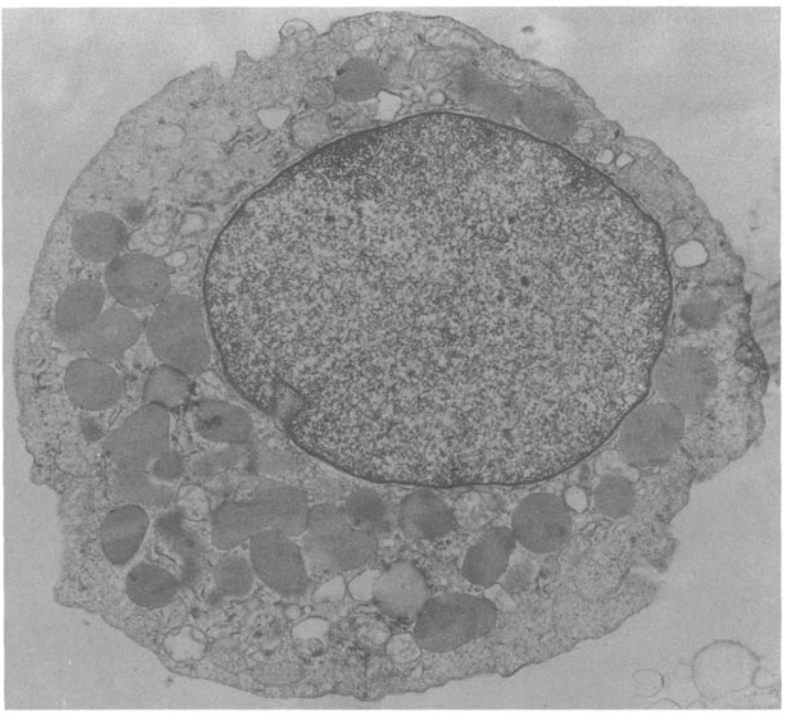

Fig. 2. Isolated juxtaglomerular cell from rat kidney. Note the numerous secretory granules. Isolation was done according to Kurtz et al. (1986b). ×16000

ful tool for studies concerning the mechanism of renin release, but these are not generally available. It was later discovered that these cultured tumoral cells do not contain typical secretory granules and that they release renin mainly in an inactive form. Recently, the same group succeeded in transforming cultured tumoral juxtaglomerular cells by the use of SV40 virus infection (Pinet et al. 1985). Although these cultures are not optimal for the study of exocytosis of renin they could be very valuable for the investigation of the control of renin and prorenin synthesis (Pinet et al. 1987).

Our own efforts concentrated on the development of a method that allows reproducible production of a cell preparation containing a high proportion of juxtaglomerular cells. Using rats we were able to produce cell preparations containing around 80% juxtaglomerular cells (Kurtz et al. 1986a, b). These cells display prominent renin granules (Fig. 2) and they spontaneously release renin, predominantly in the active form (Kurtz et al. 1986a). Moreover, the renin release can be modulated by a variety of agents that typically regulate renin release in the kidney (Kurtz et al. 1984, 1986a). Utilizing a similar technique, Johns et al. (1987) recently established cell preparations with high

renin content, and it was confirmed that the preparations are also suitable for the study of renin secretion a cellular level.

2.2 Processing of Renin in Juxtaglomerular Cells

Present knowledge of the intracellular synthesis of renin is summarized in Fig. 3. The molecular biology has recently been reviewed in detail (Dzau et al. 1988), and the structure of the human renin gene has already been eluci-

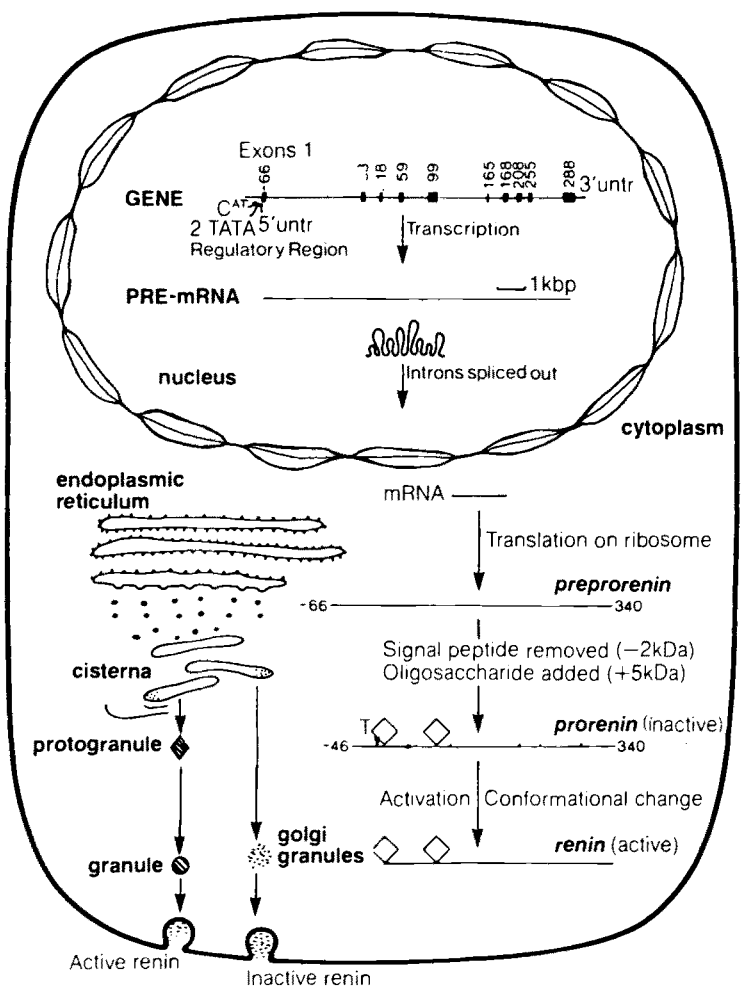

Fig. 3. Biosynthetic pathway of active renin in a juxtaglomerular cell from its gene to its release into the blood stream. (From Vallotton 1987)

dated (Hardman et al. 1984; Horbart et al. 1984). Human renal renin is encoded by a single gene which consists of ten exons and nine introns. The messenger (m) RNA of the renin gene is translated into a protein preprorenin consisting of 401 amino acid residues (Imai et al. 1983; Pratt et al. 1983). In the endoplasmic reticulum a 20-amino-acid signal peptide is cleaved from preprorenin, leaving prorenin a glycosylated protein of 381 amino acids (Panthier et al. 1982; Cantanzaro et al. 1983; Galen et al. 1984). Prorenin, which is enzymatically inactive, is packed into secretory granules at the Golgi apparatus; where it is further processed into active renin by the cleavage of a 46-amino-acid peptide from the N-terminus of the molecule. Mature renin is a monomeric glycosylated carboxypeptidase, with a molecular weight of around 44000 daltons. In addition, the structure of the renal renin gene in rats has been clarified (Burnham et al. 1987; Fukamizu et al. 1988).

Two greatly different morphological forms of granules with renin immunoreactivity can be distinguished within juxtaglomerular cells (Barajas 1966; Taugner et al. 1984). It is thought that only mature granules contain active renin. The subcellular classification of renin-containing granules is still a matter of debate. Two main types of cellular granules originate from the Golgi apparatus: the first is the typical secretory granules, which contain material to be released from the cell by an exocytotic process, and the second is lysosomes. These granules contain proteolytic and other hydrolytic enzymes. The content of lysosomes is usually bound to remain within cells and to catalyze autolytic processes. The mechanism that causes secretory granules to fuse with the cell membrane but allows lysosomes to remain within the cytoplasm is not clearly understood. Morphological differences between secretory granules and lysosomes exist. In contrast to secretory granules, lysosomes have autophagic capabilities (De Duve 1983). In addition, the lipid content of lysosomal membranes can be enhanced by so-called lysosomotropic substances such as chlorphentermine and chloroquine whilst that of typical secretory granules cannot (Houban et al. 1972; Hillmann-Rauch 1975).

Renin has been found to originate from the Golgi apparatus, like typical secretory products (Pratt et al. 1983). Since renin is a proteolytic enzyme, and such enzymes are normally stored within lysosomes, the possibility that renin-containing granules might be related to lysosomes has been considered for some time (Fisher 1966). This speculation has furthermore been confirmed by the discovery that typical lysosomal enzymes such as acid phosphatase (Fisher 1966; Ruyter 1964) and β-glucuronidase (Gomba and Soltesz 1969) coexist with renin in the granules. More recently, renin granules have been shown to display autophagic capabilities, and they also gradually accumulate polar lipids after administration of lysosomotropic substances (Taugner et al. 1985a). Overall, present knowledge of the nature of renin-containing granules within juxtaglomerular cells supports the idea that the renin granule is not a typical secretory granule, but rather resembles a modified lysosome.

Information on the intracellular control of active renin production is rather scarce. At least three regulatory steps in the production of mature renin can be identified, namely, the transcription rate of the gene, the translation rate of mRNA, and the rate of conversion of prorenin into renin. The proteolytic mechanism by which prorenin is processed into renin is not clearly understood. In view of the findings that cathepsins are capable of converting prorenin to renin in vitro (Takahashi et al. 1982), and that cathepsins B and D coexist with renin in secretory granules (Taugner et al. 1985b, 1986), it might be speculated that these cathepsins have an important function in the intracellular activation of renin. For completness, it should also be mentioned that kallikrein has been considered as a possible activator of prorenin (Yokosawa et al. 1979).

At present it is not known whether the intragranular cleavage of prorenin is subject to physiological regulation. There are, however, findings indicating that both the transcription rate of the renin gene and the translation rate of the renin mRNA are subject to physiological regulation. It is well established that states of low sodium intake into the organism are accompanied by high levels of plasma renin (Keeton and Campbell 1981). Using rats, Nakamura et al. (1985) have shown that sodium depletion and converting enzyme inhibition led to a 46-fold increase in plasma renin activity a 1.5-fold increase in renal renin activity, and a 2.8-fold increase in renal renin mRNA content compared with normal animals. The latter finding indicates that the gene transcription rate is subject to control, and assuming that the biological half-life of plasma renin is not altered in states of low sodium intake, it follows from the results of these experiments that the translation rate of a single renin mRNA molecule is also enhanced in this situation. This conclusion is in agreement with the results obtained by Morimoto et al. (1979), who found that low sodium intake in rats caused a 12-fold increase in plasma renin activity and a 3-fold increase in granular renin activity. Further confirmation of transcription and translation regulation was recently obtained by Moffet et al. (1986), who found that aorta coarctation led to a 50-fold increase in renin mRNA and renin enzyme activity in the ischemic kidney. However, identification of the cellular factors that control transcription and translation of the renin gene remains a task for future research.

2.3 Mechanisms of Renin Secretion

Components of the cytoskeleton, e.g., microtubules and microfilaments, are necessary for cell motility and intracellular translocation processes (Trifaro et al. 1985). Since a typical secretory process requires the movement of secretory granules from within the cell to the cellular plasma membrane, there is speculation that the cytosolic part of the cytoskeleton is involved in this process

(Lacy et al. 1968; Sherline et al. 1977; Hoffstein and Weissmann 1978). The subplasmalemmal part of the cytoskeleton, however, is considered to have an inhibitory role in secretion (Burgoyne et al. 1987, 1988). Significant alterations in the arrangement of microfilaments in the space between the secretory granule and the cellular membrane have been observed upon secretion (Hall 1982; Boyd 1982). Moreover, it has been found that the typical secretory process in a variety of secretory cells can be altered by substances which interfere with the normal assembly of microtubules and microfilaments (Hall 1982; Boyd 1982; Perrin et al. 1987). In view of these findings the question arises of the role of the cytoskeleton in the secretion of renin granules from juxtaglomerular cells.

Juxtaglomerular cells have been found to be rather poor in their content of microfilaments and microtubules (Taugner et al. 1984). No significant alteration in the arrangement of microfilaments upon secretion of renin has been observed in juxtaglomerular cells (Taugner et al. 1984). Furthermore, it has been demonstrated in whole kidneys and in kidney slices that colchicine, which interferes with the normal assembly of microtubules, has no inhibitory effect on the secretion of renin from juxtaglomerular cells (Hackenthal et al. 1978). In our own studies using isolated juxtaglomerular cells and colchicine we did not observe alterations in spontaneous renin release (Kurtz et al. unpublished).

Since there is no positive evidence that the microfilament system plays an active role in the extrusion of renin, it is uncertain whether renin release requires translocation of secretory granules at all. In fact, it has been suggested that renin could be released from storage granules into the cytoplasm of juxtaglomerular cells, and then may pass through the plasma membrane. This idea was first discussed by Rouiller and Orci (1971), and then later revived by Fray et al. (1983 a, b) and Barajas and Powers (1984). Experimental evidence, however, is rather limited, and is based on the finding that isolated renin granules release more renin in the presence of a low calcium concentration (Sagnella and Peart 1979). Since a fall in the cytosolic calcium concentration is believed to be a stimulatory signal for renin release (see Sect. 3.3), it has been hypothesized that a fall in cytoplasmic calcium sets in motion the release of renin from the granules into the cytoplasm and finally across the plasma membrane. Recently, Zavagli et al. (1983) reported morphological evidence of cytoplasmic solubilization of renin granules. Support for this mechanism of renin release was also provided by Fray and Lush (1984), who found that upon stimulation of renin release the renin activity increased in the cytosolic phase of a kidney extract, whilst that of the granular fraction remained unchanged. It should be mentioned, however, that the experimental protocol used in this study did not allow cytoplasmic renin and extracellular renin which might have been released by exocytosis to be clearly distinguished.

In contrast, there is strong morphological evidence that renin release from juxtaglomerular cells occurs as an exocytotic process involving fusion be-

tween the membranes of the secretory granule and the plasma membrane (Taugner et al. 1984). But how is the contact between these two membranes brought about in juxtaglomerular cells? Peter (1976) obtained morphological evidence of deep, channel-like invaginations of the plasma membrane in rat juxtaglomerular granulated cells. He inferred that renin secretion represents an unusual type of exocytosis in which the plasma membrane invaginates towards the granules, thus providing sites for extrusion, instead of the granules moving towards the cell surface prior to release. This idea was supported by the findings of Ryan et al. (1982), which showed that the incidence of these deep invaginations increases during stimulus induced degranulation and decreases during regranulation of juxtaglomerular cells. Recent electron-microscopical studies performed by Taugner et al. (1984), however, show that granules lying next to the juxtaglomerular cell membrane change their shape upon stimulation of secretion by protruding towards the cell membrane, but the reason for this is not clear. Morphological evidence indicates that structural alterations within the renin granule accompany this protrusion process (Taugner et al. 1984). The authors speculated that these intragranular alterations are due to changes in osmolarity of the granules; however, the mechanism for these changes of intragranular osmolarity upon stimulation of secretion in juxtaglomerular cells is not known.

Taugner et al. (1987) obtained morphological evidence showing that not only mature granules but also juvenile granules, which contain mostly prorenin fuse with the juxtaglomerular cell membrane. This observation could explain the origin of prorenin in the plasma. In humans, for instance, prorenin can account for up to 90% of the total amount of renin present in the plasma (Sealy et al. 1986). Further evidence that renin is released by an exocytotic process was provided by Skott (1986). Using isolated afferent arterioles, he observed a quantum-like release of renin from these arterioles. This mode of renin release could be explained by the exocytosis of single secretory granules containing renin.

In summary, it appears likely that renin is released from renal juxtaglomerular cells by an exocytotic process which involves fusion of the granule's membrane and the cellular plasma membrane. However, the mechanism, by which the contact between the two membranes is brought about is not yet clear.

3 Intracellular Signals for the Control of Renin Secretion

3.1 General Remarks

In recent years it has become evident that the phosphatidylinositol cycle (PI cycle) plays a major role in the stimulus-secretion coupling of secretory cells

(Berridge 1984). Stimulators of secretion usually activate a specific plasma membrane-bound phosphodiesterase, phospholipase C (PLC) which splits phosphatidylinositol-4,5-bisphosphate into diacylglycerol (DAG) and inositol triphosphate (IP_3). IP_3 releases calcium from intracellular stores in the endoplasmic reticulum, and consequently leads to a rise in the intracellular concentration of calcium (Streb et al. 1983), while DAG stimulates a phospholipid-dependent protein kinase C (Nishizuka 1984). There is good experimental evidence to indicate that exocytosis in a typical secretory cell is initiated or facilitated by the rise in calcium induced by IP_3 (Penner and Neher 1988). The secretory response is then maintained, or even enhanced, both by the activation of the C kinase by DAG and by an increased transmembrane calcium influx caused by the opening of calcium channels in the plasma membrane (Rasmussen 1986; Alkon and Rasmussen 1988). Activation of voltage-gated calcium channels is an additional process by which secretion from excitable cells is triggered.

In view of the importance of calcium and C kinase activity it is obvious to consider the role of these in the control of renin release from juxtaglomerular cells.

3.2 Regulation of Calcium in Renal Juxtaglomerular Cells

Only limited information exists on the regulation of calcium within juxtoglomerular cells, mainly due to the paucity and difficult accessibility of these cells. Recently a combination of patch clamp and calcium microfluorimetry was utilized to gain insight into the regulation of calcium within single juxtaglomerular cells (Kurtz and Penner 1989). The results obtained indicate that juxtaglomerular cells contain intracellular stores of calcium which can be mobilized by receptor-operated and G-protein-mediated stimulation of PLC activity.

It is interesting that activation of PLC sets in motion oscillations of intracellular calcium (Fig. 4). The frequency of these oscillations is positively correlated to the extracellular concentration of calcium. Moreover, calcium release in juxtaglomerular cells is dependent on the membrane potential; membrane hyperpolarization facilitates calcium release and membrane depolarization blocks calcium release. Clear evidence for hormone-induced transmembrane calcium influx has been found (Kurtz and Penner 1989; Kurtz et al. 1986a). In this study no positive evidence for the existence of voltage-operated calcium channels (VOCC) was obtained. The presence of VOCC in juxtaglomerular cells, however, cannot be definitively ruled out as a result of this study, because it is known that VOCC can display very fast run-down kinetics in vitro (Tanita 1988).

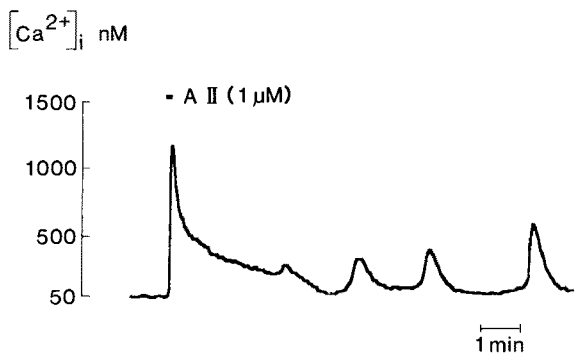

Fig. 4. Oscillations of intracellular calcium in renal juxtaglomerular cells induced by angiotensin II (1 μ*M*). (Adapted from Kurtz and Penner 1989)

3.3 Calcium

Although there are some individual findings suggesting a positive role for calcium in renin release (Morimoto et al. 1970; Iwao et al. 1974; Lester and Rubin 1977; Ginesi et al. 1981), much evidence argues in favor of an inhibitory role. Results obtained from experiments with whole kidneys, renal cortical slices, renal glomeruli, and isolated juxtaglomerular cells indicate that the rate of renin release is inversely related to the extracellular concentration of calcium (Kisch et al. 1976; Watkins et al. 1976; Fray 1977; Kotchen et al. 1977; Harada and Rubin 1978; Fray and Park 1979). Since the intracellular and extracellular calcium concentrations are positively correlated (Kurtz and Penner 1989) the rate of renin release is expected to be inversely related to the intracellular concentration of calcium as well.

Additional information on the role of calcium in the intracellular regulation of renin release was gained from experiments in which the calcium extrusion from juxtaglomerular cells was blocked. Calcium extrusion from a cell is generally brought about by two processes: (1) a sodium-calcium exchange mechanism (Baker 1976), and (2) energy-dependent calcium adenosine triphosphatase (ATPase) (Baker 1976; Rasmussen and Barret 1984). The sodium-calcium exchange is driven by the sodium gradient between the extracellular and intracellular spaces (Blaustein 1974). Since this gradient is maintained by the activity of sodium-potassium ATPase, the function of the sodium-calcium exchange is linked to the activity of the sodium-potassium ATPase. Consequently inhibition of sodium-potassium ATPase by ouabain blocks the sodium-calcium exchange. Therefore, ouabain leads to an accumulation of calcium within the cell and consequently to a rise in the intracellular calcium concentration. Ouabain has been found to inhibit renin release (Lyons and Churchill 1974; Park and Malvin 1978; P. C. Churchill 1979; Part et al. 1981; P. C. Churchill and M. C. Churchill 1982a), and this inhibition has been shown to be a calcium-dependent process (Park and Malvin 1978; P. C. Churchill 1979; Cruz-Soto et al. 1984). Inhibition of calcium ATPase by vanadate

partly inhibits the extrusion of calcium, and therefore leads to a rise in the intracellular calcium concentration (O'Neal et al. 1979). Vanadate has also been found to inhibit renin release (P. C. Churchill and M. C. Churchill 1980; Lopez-Novoa et al. 1982). These findings suggest that a rise in the intracellular calcium concentration inhibits the secretory process in the juxtaglomerular cells.

It has been observed that spontaneous renin release from whole kidneys, kidney slices, glomeruli, and isolated juxtaglomerular cells is inhibited by facilitation of calcium entry (Flynn et al. 1977; P. C. Churchill and M. C. Churchill 1982a, b, 1987; M. C. Churchill et al. 1983; Matsumara et al. 1984; Raguki et al. 1988), and is enhanced by calcium entry blockers (P. C. Churchill et al. 1981; Abe et al. 1983; Kurtz et al. 1984; Antonipillai and Horton 1985). Inhibition of calcium release from intracellular stores has also been reported to enhance renin release (Fray and Lush 1984). In addition, calcium mobilizing hormones such as angiotensin II, vasopressin, norepinephrine, and platelet activating factor have been found to inhibit renin release by a calcium-dependent process (Vandongen and Peart 1974; Vandongen 1975; P. C. Churchill 1981; Kurtz et al. 1984; Pfeilschifter et al. 1985). Using isolated juxtaglomerular cells, it was shown that these hormones also enhanced transmembrane calcium influx into the cells (Pfeilschifter et al. 1985; Kurtz et al. 1986a), and that renin release from juxtaglomerular cells is inversely correlated to the transmembrane calcium influx. We and others have shown that the inhibitory effect of these calcium mobilizing agents can be attenuated, or even abolished, by calcium channel blockers such as verapamil (P. C. Churchill 1980; Part et al. 1981; Kurtz et al. 1986a).

In summary, all the findings mentioned above support the idea that the rate of renin release from juxtaglomerular cells is *inversely* related to the calcium concentration in these cells. Thus renin secretion behaves strikingly differently from the typical secretory mechanism in which the secretion rate is positively correlated to the intracellular calcium concentration (Rasmussen 1986). The mechanism by which intracellular calcium inhibits the secretory process in juxtaglomerular cells is still unknown.

Calcium-dependent reactions are usually triggered by calcium in two ways, either by the action of ionized calcium, or by calcium-binding proteins. Calmodulin is one of the most important calcium-binding proteins in smooth muscle cells with respect to its quantity and function. The question as to whether the inhibitory effect of calcium on renin release could be mediated by calmodulin has been addressed by investigating the effect of calmodulin antagonists on renin release. Indeed, calcium calmodulin antagonists stimulate renin release from rat glomeruli (Kawamura and Inagami 1983), rat renal cortical slices (P. C. Churchill and M. C. Churchill 1983; Matsumara et al. 1984; Antonipillai and Horton 1985; Part et al. 1986), isolated perfused rat kidneys (Fray et al. 1983b; Schwertschlag and Hackenthal 1983), and isolated

juxtaglomerular cells (Kurtz et al 1984). However, there is some concern that these findings may not really prove an essential role for calmodulin in the inhibition of renin secretion by calcium. For instance, we found in our studies that trifluoperazine, which is the most commonly used calmodulin antagonist in the study of renin secretion, also inhibits the transmembrane calcium influx into the juxtaglomerular cells (Kurtz et al. 1984), a fact that is also known from studies on other tissues (Seeman 1972). It is, therefore, difficult to decide from these experiments whether the stimulation of renin secretion is due to calmodulin inhibition, or to inhibition of calcium influx. The calmodulin antagonist W7, which apparently does not affect the calcium conductance of the cell membrane, was also reported to stimulate renin release (Matsumara et al. 1984); however, inhibition of cyclic adenosine monophosphate (cAMP) phosphodiesterase cannot be excluded. Since cAMP is considered to be a stimulatory signal for renin release (see Sect. 3.5) it is not definitely clear from this study whether or not inhibition of calmodulin is the reason for the stimulation of renin release by W7.

In summary then, although there is much evidence that calmodulin antagonists stimulate renin release, clear proof that calmodulin mediates the inhibitory effect of calcium on renin secretion is still lacking.

3.4 Protein Kinase C Activity

As mentioned previously, the exocytotic process in a number of secretory cells is initiated by a rise in the intracellular calcium concentration and is sustained of facilitated by the activity of protein kinase C. These intracellular events are normally caused by the activation of plasma membrane-bound phospholipase C (PLC), which splits polyphosphoinositides into inositol phosphates and diacylglycerol (DAG) (Berridge 1984). DAG is considered to be the physiological intracellular activator of protein kinase C (Nishizuka 1984).

It has been shown that hormones which cause breakdown of phosphatidylinositol bisphosphate in isolated juxtaglomerular cells such as angiotensin II, arginine-vasopressin, norepinephrine, and platelet activating factor increase the content of DAG within the cells, suggesting stimulation of protein kinase C by these hormones (Pfeilschifter et al. 1985; Kurtz et al. 1986a). These hormones also inhibit renin release from the cells. Since activation of PLC causes both calcium mobilization and activation of protein kinase C, and since calcium mobilization is considered to be an inhibitory signal for renin release, it is difficult to evaluate the role of protein kinase C in these experiments. Direct stimulation of protein kinase C activity can be achieved by tumor-promoting phorbol esters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Castagna et al. 1982). TPA, when added either to isolated juxtaglomerular cells (Kurtz et al. 1986a, b, c) or to renal cortical slices (Churchill and Churchill

1984), leads to a dose-dependent inhibition of renin release; a significant effect, however, was only observed with rather high concentrations of TPA. At high concentrations TPA is also suspected of acting as a calcium ionophore, independently of its activating effect on C-kinase (Castagna et al. 1982). Since an enhanced calcium influx brought about by a calcium ionophore would be expected to inhibit renin release, a direct inhibitory effect by TPA on renin release cannot be excluded. Churchill and Churchill (1984), however, provided indirect evidence that the inhibitory effect of TPA on renin release from kidney slices was not due to an ionophore effect. In our own studies we have found that TPA enhances transmembrane calcium influx into isolated juxtaglomerular cells (Kurtz et al. 1986a). It is, therefore, not yet clear whether TPA inhibits renin release by the phosphorylation of as yet unknown proteins via C kinase activation or by enhancing calcium influx independently of C kinase activation.

Despite the uncertainty about the way phorbol esters affect renin release, it can at least be said that activation of C kinase does not stimulate the secretory process in juxtaglomerular cells. In this respect, the secretory mechanism in juxtaglomerular cells seems to differ significantly from that of other endocrine and exocrine cells.

3.5 Cyclic Nucleotides

3.5.1 General Remarks

Cyclic nucleotides, in particular cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), are capable of modulating the secretory response initiated and maintained by calcium and protein kinase C activity.

Activation of adenylate cyclase is known to be an important signal for the regulation of secretion in different types of secretory cells. In contrast to the effect of calcium, the effect of cAMP on the secretion process is not uniform (Rasmussen and Barrett 1984); cAMP can either enhance the secretory response, e.g., in pancreatic cells), or even decrease the secretory response, e.g., in platelets or mast cells (Penner and Neher 1988). The mechanism by which cAMP affects secretion in these cells has not been elucidated in detail, but it seems likely that cAMP exerts its effect by the activation of a cAMP-dependent protein kinase. Moreover, there is growing evidence that cAMP affects the secretion process by interfering with calcium and C kinase activity.

Recent findings indicate that cGMP is capable of inhibiting the secretory response in some cell types. Matsuoka et al. (1985) have shown the inhibition of aldosterone secretion from adrenal glomerulosa by atrial natriuretic peptide is associated with an increase in cGMP production. In addition, it was

demonstrated that cGMP attenuates exocrine pancreatic secretion stimulated by cholecystokinin (Otsuki et al. 1986) or protein kinase C (Rogers et al. 1988).

In view of the effects of cyclic nucleotides on the secretory response in typical secretory cells, possible effects on the control of renin release should be considered.

3.5.2 Role of cAMP

There is broad evidence that substances which activate adenylate cyclase such as β-catecholamines (Davis and Freeman 1976; Keeton and Campbell 1981), prostaglandins (Gerber et al. 1978; Hackenthal et al. 1980; Weber et al. 1976; Wernig et al. 1971), glucagon (Udea et al. 1978; Vandongen et al. 1973), and parathyroid hormone (Powell et al. 1978; Smith et al. 1979) stimulate renin release. Application of membrane-permeable cAMP analogs also enhances renin secretion (Peart et al. 1975; Viskoper et al. 1977). Furthermore, inhibition of cAMP phosphodiesterase leads to stimulation of renin release (Peart et al. 1975; Viskoper et al. 1977). These findings were obtained using isolated perfused kidneys, kidney slices, isolated renal glomeruli, and isolated juxtaglomerular cells. Using isolated juxtaglomerular cells, we showed that activators of adenylate cyclase such as isoproterenol, prostacyclin, and forskolin increase the cellular content of cAMP (Fig. 5) (Kurtz et al. 1984).

The question arises by which mechanism cAMP stimulates renin release. In particular, it must be considered whether cAMP influences renin release directly or indirectly by decreasing the intracellular calcium concentration. Using isolated juxtaglomerular cells, we obtained evidence that the rate of renin release is correlated to the intracellular level of cAMP in the presence of activators of adenylate cycles. However, an increase in the level of cAMP was found not to be a prerequisite for the stimulation of renin release. In the presence of the calcium channel blocker verapamil and the calmodulin antagonist trifluoperazine we observed a two- to threefold increase in the rate of renin release a figure that is comparable with the effects of isoproterenol and prostacyclin on renin secretion. Verapamil and trifluoperazine, however, had no effect on the cellular cAMP level. Agents known to inhibit renin release such as angiotensin II did not influence the cAMP level (Kurtz et al. 1984). These findings suggest that cAMP is at least not the dominant regulator of renin release from juxtaglomerular cells.

Four lines of experimental evidence suggest that cAMP may stimulate renin release by interference with the intracellular concentration of calcium in juxtaglomerular cells:

1. It has been shown that cAMP inhibits calcium mobilization from intracellular stores in single juxtaglomerular cells (Kurtz and Penner 1989).

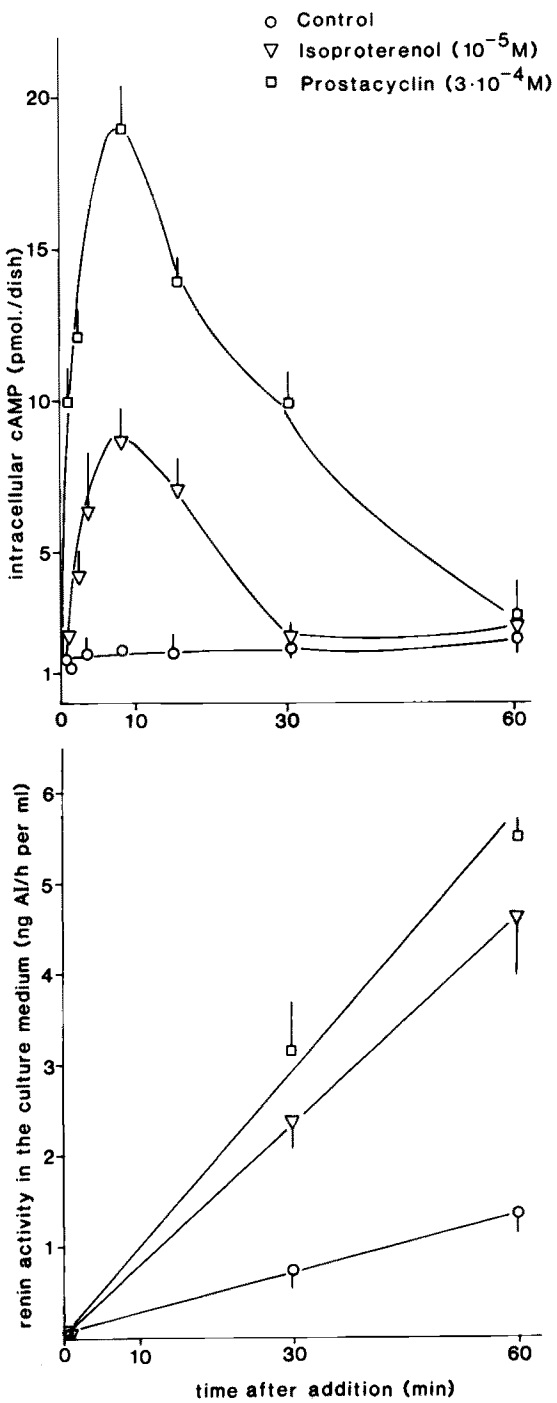

Fig. 5. cAMP levels in isolated rat renal juxtaglomerular cells (*upper panel*) and renin activity measured by angiotensin I (*AI*) production (*lower panel*) after addition of prostacyclin and isoproterenol

2. In experiments using isolated juxtaglomerular cells it was found that activators of adenylate cyclase decrease the calcium permeability of the plasma membrane (Kurtz et al. 1984).
3. It has been shown that the stimulatory effect of cAMP can be attenuated or even abolished by maneuvers which are expected to increase the intracellular concentration of calcium (Churchill and Churchill 1982a; Opengorth and Zehr 1983; Vandongen 1975).
4. It has been found that cAMP is capable of stimulating calcium ATPase and the sodium-calcium exchange mechanism in a variety of cells (Phillis and Wu 1981; van Breemen et al. 1979), both processes are expected to cause a fall in the intracellular concentration of calcium. Altogether, there are good reasons to argue that cAMP and activators of adenylate cyclase may stimulate renin release by decreasing intracellular calcium activity.

3.5.3 Role of cGMP

Little information exists on the possible role of cGMP in the control of renin release. Recently, a hormone has been discovered that is thought to act on its target cells by increasing the intracellular level of cGMP. This hormone is the atrial natriuretic peptide (ANP) (Ballermann and Brenner 1985), and besides its natriuretic effect, it has also been found to inhibit renal renin release (Burnett et al. 1984; Garcia et al. 1985; Maack et al. 1985). It was not clear whether ANP exerts its effect by a direct action on juxtaglomerular cells, or indirectly by activating the macula densa mechanism; therefore, the effects of ANP, on renin release from renal cortical slices (Obana et al. 1985; Henrich et al. 1986; Takagi et al. 1988; Antonipillai et al. 1986) and from rat (Kurtz et al. 1986b) and tumoral human juxtaglomerular cells (Pinet et al. 1987) were investigated. With the exception of one study (Tagaki et al. 1986), the results revealed that ANP leads to a dose-dependent inhibition of renin release from these preparations. ANP also caused enhanced release of cGMP and decreased release of cAMP from perfused kidney preparations (Obana et al. 1985).

The observation that ANP is capable of stimulating guanylate cyclase (Tremblay et al. 1985) and simultaneously inhibiting adenylate cyclase in the kidney (Anand-Srivasta et al. 1984) provoked the question of whether the inhibitory effect of ANP on renin release was due to the rise in cGMP or to the decrease in cAMP. Using isolated juxtaglomerular cells we obtained clear evidence that the inhibition of renin release by ANP correlates with the level of cGMP and not with the level of cAMP (Kurtz et al. 1986b). Moreover, we found that renin release from isolated juxtaglomerular cells is inversely related to the cellular level of cGMP. Pinet et al. (1987) also provided evidence that the inhibitory effect of ANP on renin release from human tumoral juxtaglomerular cells is not mediated by cAMP. From these findings it may be

inferred that ANP inhibits renin release by raising the intracellular level of cGMP. This may indicate that cGMP is an inhibitory signal in the control of renin release in juxtaglomerular cells, a conclusion supported by the observation that renin release from renal cortical slices (Hirume et al. 1986) and isolated juxtaglomerular cells (Kurtz et al. 1986b) is inhibited by nitroprusside, which is a well known activator of soluble guanylate cyclase. Recently, it was demonstrated that endothelium-derived relaxing factor, which acts by stimulating soluble guanylate cyclase, also inhibits renin release in vitro (Vidal et al. 1988). In addition, membrane-permeable cGMP analogs such as 8-bromo-cGMP have been found to inhibit renin secretion from renal cortical slices (Henrich et al. 1988; Hiruma et al. 1986) and isolated juxtaglomerular cells (Kurtz et al. 1988c).

As with cAMP, the question arises as to whether cGMP has a direct effect on the regulation of renin release or whether it acts indirectly by increasing the intracellular calcium concentration. Calcium measurements in single juxtaglomerular cells revealed that cGMP does not alter basal calcium levels and does not interfere with calcium mobilization. Moreover, it was shown that ANP, which is thought to act via cGMP, did not alter calcium-45 uptake into isolated juxtaglomerular cells. It is likely, therefore, that the inhibitory effect of cGMP on renal renin release does not involve an increase in the cytosolic calcium concentration. It should be noted that in studies using isolated juxtaglomerular cells the inhibitory effect of ANP on renin release was markedly attenuated in the presence of the calcium channel blocker, verapamil (Kurtz et al. 1986c). Since verapamil decreases the basal calcium influx into the cells (Kurtz et al. 1984; 1986a), one could assume from these findings that cGMP requires a normal calcium concentration to exert its maximal inhibitory effect on renin release.

The available experimental evidence thus indicates that cGMP is an inhibitory signal for renin release from juxtaglomerular cells and that it does not affect intracellular calcium concentration.

3.6 G Proteins

The role of GTP-binding (G) proteins in the control of exocytosis has gained increasing interest. In particular, three types of G proteins are considered to be involved in the regulation of secretion:

1. A G protein, G_P, which couples cell surface receptors to PLC (Burgoyne 1987). This type of G protein thus mediates receptor mediated calcium release and activation of protein kinase C.
2. A G protein which is involved in secretion, G_E (Burgoyne 1987), the existence of which has been inferred from experiments on mast cell secretion

(Barrowman et al. 1986). The detailed mechanism of action of this G protein has not yet been elucidated.

3. A type of G protein which recent findings (Axelrod et al. 1988) have suggested may mediate receptor-operated activation of phospholipase A_2 (PLA_2): some evidence indicates that PLA_2 activity could also be involved in the regulation of secretion (Burgoyne et al. 1987).

So far only indirect evidence exists about the role of G proteins in the cellular regulation of renin secretion from juxtaglomerular cells. This evidence is based on results from experiments with pertussis toxin (PT), which is known to inhibit certain G proteins.

It has been found that PT enhances basal renin release in the intact organism (Pedraza-Chaveri et al. 1985), isolated perfused kidney (Hackenthal et al. 1987), renal cortical slices (Pedraza-Chaveri et al. 1986; Rossi et al. 1987), and isolated juxtaglomerular cells (Kurtz et al., unpublished). From these findings it may be inferred that PT-sensitive G proteins in juxtaglomerular cells act as inhibitory signals for renin secretion.

In addition, it was shown that PT blunted the inhibitory effects of angiotensin II (Hackenthal et al. 1985), noradrenergic agonists (Pedraza-Chaveri et al. 1986), neuropeptide Y (Hackenthal et al. 1987), and adenosine (Rossi et al. 1987) on renin secretion. It was speculated that these hormones all inhibit renin secretion via the G protein which inhibits adenylate cyclase (N_i), because this G protein is the classic target of PT (Murayama and Ui 1983). Moreover, blocking the inhibition of cAMP formation in juxtaglomerular cells could explain the observed stimulatory effect of PT on renin secretion. However, recent evidence indicates that PT is also capable of inhibiting G proteins other than N_i in a number of cell types (Axelrod et al. 1988). The calcium mobilizing effects of angiotensin II are mediated by G proteins in juxtaglomerular cells (Kurtz and Penner 1989).

At this point, some attention should be paid to the role of PLA_2 activity in the control of renin secretion. Most of the conditions known to inhibit renin secretion are associated with a stimulation of PLA_2 activity and vice versa. Specifically, vasoconstrictive hormones (Ardaillou et al. 1985) and elevation of intracellular calcium (Hassid 1982) are known to stimulate PLA_2 activity and to inhibit renin secretion. In contrast, inhibition of PLA_2 activity by low calcium, cAMP (Hassid 1982), calmodulin antagonists (Craven and De Rubertis 1983), and cyclosporin A (Kurtz et al. 1987) is associated with stimulation of renin secretion from juxtaglomerular cells (Kurtz et al. 1988b). The possibility that PLA_2 plays an inhibitory role in renin secretion is supported by the recent findings that derivatives of arachidonic acid, which is liberated by PLA_2 activity, are inhibitory for renin secretion (Antonipillai et al. 1987), and could mediate the inhibitory effect of angiotensin II on renin secretion (Antonipillai et al. 1988).

In summary, there is indirect evidence that G proteins play an inhibitory role in renin secretion. The nature of the G proteins possibly involved in the regulation of renin secretion still has to be clarified.

3.7 Membrane Potential

Electrical processes are known to have an important function in the regulation of exocytosis from secretory cells (Petersen and Maruyama 1984; Penner 1988). Before discussing the possible involvement of ion channels in the regulation of renin secretion, the general electrical properties of juxtaglomerular cells should be considered. Microelectrode studies have revealed a rather negative membrane potential ranging from −60 to -80 mV (Fishman 1976; Bührle et al. 1985). These findings were confirmed by recent patch clamp studies on single juxtaglomerular cells (Kurtz and Penner 1989). The negative membrane potential results from an anomalous inward-rectifying potassium current (Kurtz and Penner 1989); this explains the strict dependence of the juxtoglomerular cell membrane potential on the extracellular concentration of potassium (Fishman 1976). In addition, a delayed rectifying potassium current which is activated upon depolarization was found. No evidence of voltage-gated sodium or calcium currents was obtained in the patch clamp study; however, the existence of large calcium-activated currents was documented (Fig. 6). This appears to be via calcium-activated chloride channels, which are directly regulated by the intracellular concentration of calcium within juxtaglomerular cells. It is tempting to speculate that these chloride channels are involved in the inhibition of renin secretion by calcium (see Sect. 4).

An increase in the extracellular concentration of potassium (Fishman 1976) and of the vasoconstrictive hormones angiotensin II, arginine-vasopressin, and norepinephrine (Bührle et al. 1986b) causes a significant depolarization of juxtaglomerular cells, and at the same time an inhibition of renin secretion (Churchill 1980; Bührle et al. 1986a, b). Due to the simultaneity of both events it was speculated that membrane depolarization is part of the mechanism by which vasoconstrictor hormones inhibit renin secretion. It has been shown that the inhibition of renin release by potassium depolarization and vasoconstrictive hormones can be significantly attenuated by calcium channel blockers (P. C. Churchill 1980; Kurtz et al. 1986a). Since membrane depolarization is the signal for the opening of voltage-dependent calcium channels, it was inferred that membrane depolarization leads to an inhibition of renin release by enhancing transmembrane calcium entry into the juxtaglomerular cells (P. C. Churchill 1980; Bührle et al. 1986b). This conclusion is apparently contradictory to the findings of patch clamp investigations, which did not provide evidence for the existence of voltage-gated calcium channels. However, the possibility that juxtaglomerular cells possess voltage-gated calcium

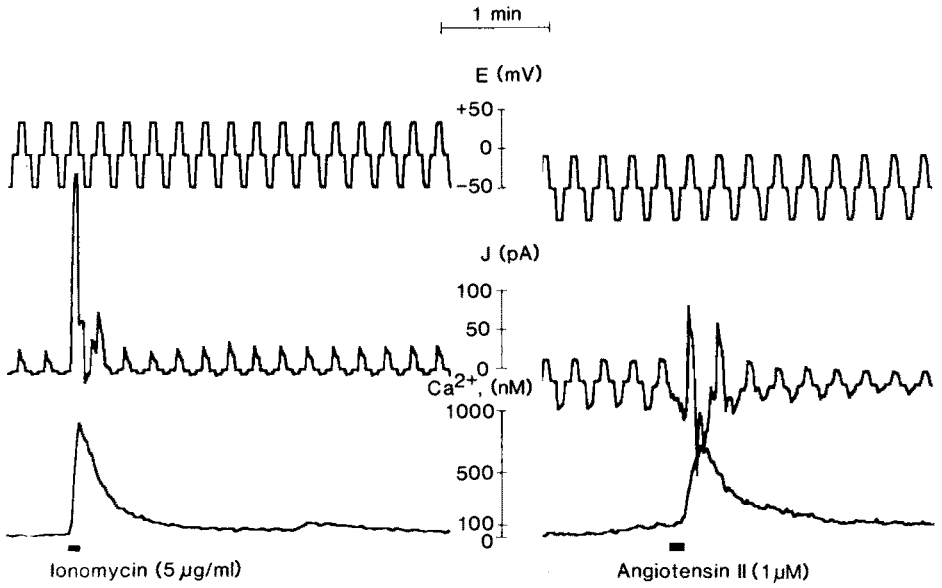

Fig. 6. Chloride currents in renal juxtaglomerular cells activated by increases in intracellular calcium evoked by ionomycin (5 µg/ml) and angiotensin II (1 µ*M*). *E*, membrane potential; *J*, current

channels with unusually fast run-down kinetics cannot be definitively ruled out as a result of the patch clamp study. This study, on the other hand, has confirmed that angiotensin II causes depolarization of juxtaglomerular cells, which most likely results from reversible inhibition of the anomalous inward-rectifying potassium current (Kurtz and Penner 1989).

The question of whether hyperpolarization of juxtaglomerular cells is a stimulatory signal for renin release cannot be answered yet. Fishman (1976) reported that epinephrine, which is known to stimulate renin secretion, induced a small hyperpolarization; however, this was not confirmed by the study of Bührle et al. (1986a, b), who observed that β-adrenergic compounds, which stimulate renin release, had no influence on the membrane potential of juxtaglomerular cells.

Recently, it was shown that Cromakalim, a drug which hyperpolarizes smooth muscle cells by the opening of certain potassium channels (Hamilton et al. 1986), stimulates renin secretion in humans in vivo and also in vitro from isolated rat renal juxtaglomerular cells (Ferrier et al. 1989). However, presently it is not known whether Cromakalim enhances renin secretion by causing membrane hyperpolarization in juxtaglomerular cells or by a different mechanism.

Overall, therefore, there are some indications that the electrical behavior of juxtaglomerular cells affects the exocytosis of renin. In particular, membrane

depolarization could cause inhibition of renin secretion. Whether this phenomenon is mediated by voltage-gated calcium channels or by different mechanisms, such as Na^+/Ca^{2+} exchange, needs to be clarified.

3.8 Osmotic Forces

Skott (1988) has recently reviewed in detail the possible role of osmotic forces in the exocytosis of renin. Changes in extracellular osmolarity are known to significantly alter exocytosis in a number of secretory cells. This behavior appears to be a rather uniform process, increase in extracellular osmolarity causing an inhibition of secretion and vice versa (see Skott 1988). Renin secretion from juxtaglomerular cells shows the same dependence in osmolarity (Skott and Taugner 1987); renin secretion appears to be very sensitive to rather small changes in osmolarity (Skott and Taugner 1987). Moreover, changes in renin secretion occurs only with a short delay after osmolarity changes.

It has been demonstrated that the nature of osmotically active particles is important for their effect on renin secretion, the effect on exocytosis is directly related to the reflection coefficient of the particle (Frederiksen et al. 1975). From this observation juxtaglomerular cells are assumed to behave as sensitive osmometers. The mechanism by which changes in extracellular osmolarity alter renin secretion from juxtaglomerular cells is not yet known, but cell volume may be an important control mechanism since it is possible that states of decreased cell volume inhibit exocytosis of renin, and vice versa (Skott 1988).

At this point it should be recalled that morphological evidence exists which shows that renin-containing granules swell before or during exocytosis (Taugner et al. 1984; Skott and Taugner 1987). If such a swelling is a prerequisite for exocytosis, then renin secretion would obviously depend on the availability of cytosolic water.

4 Possible Explanations for the "Calcium Paradox"

Having considered the influence of a number of intracellular parameters on renin secretion from juxtaglomerular cells, the question arises as to whether or not the individual findings allow the development of a more general concept of the cellular control of renin secretion. Although this is not entirely possible, some guidelines can be recognized. Thus, calcium appears as the dominant second messenger for renin secretion, while C kinase, cAMP, and cGMP either act via calcium, or at least require calcium to exert their effects. Calcium has an inhibitory and therefore "paradoxical", effect on secretion

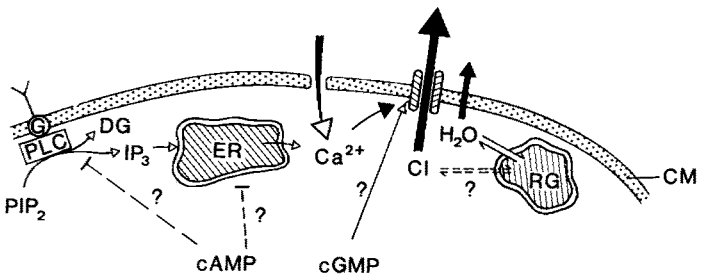

Fig. 7. Possible role of calcium-activated chloride channels in the regulation of renin secretion. *G*, GTP-binding protein; *PLC*, phospholipase C; *PIP_2*, phosphatidylinositol bisphosphate; *DG*, diacylglycerol; *IP_3*, inositol triphosphate; *ER*, endoplasmic reticulum; *RG*, renin granule; *CM*, cell membrane

from juxtaglomerular cells. Calcium in juxtaglomerular cells appears to be regulated by transmembrane influx, release from intracellular stores, calcium ATPase, and sodium/calcium exchange. Moreover, it seems that the calcium concentration just beneath the plasma membrane is more important for the regulation of renin secretion than the cytosolic calcium concentration. Based on this consideration some speculations have been made as to how calcium inhibits renin secretion.

Taugner et al. (1988) observed that the myofibrils in juxtaglomerular cells are displaced to a small subplasmalemmal border, and they developed the hypothesis that the calcium-dependent state of contraction of these myofilaments regulates the exocytosis; increased subplasmalemmal calcium concentration could cause myofilaments to form a shield between the granules and the plasma membrane, thus inhibiting exocytosis. This concept is compatible with the idea that the subplasmalemmal portion of the cytoskeleton has a general inhibitory function in secretion (Burgoyne et al. 1988). The concept could also explain the apparent stimulatory effect of cAMP on renin secretion, because cAMP is expected to inhibit the contraction of the myofibrils, both by inhibition of calcium mobilization and by inactivation of myosin light chain kinase (Adelstein 1983).

A different explanation as to how calcium could regulate renin secretion is based on the existence of calcium-activated chloride channels (Fig. 7). The activity of the chloride channels is directly regulated by the subplasmalemmal concentration of calcium. The sum conductance of these chloride channels is very large in juxtaglomerular cells (Kurtz and Penner 1989), and it can be assumed that the channels contribute significantly to the regulation of cell volume juxtaglomerular cells. Since the membrane potential in juxtaglomerular cells is close to the potassium equilibrium potential, and thus far more negative than the chloride equilibrium potential, a calcium-regulated chloride efflux from juxtaglomerular cells can be predicted. Chloride ions would be

accompanied by potassium via voltage-gated inward-rectifying potassium channels. The efflux of potassium chloride would cause cell shrinkage and consequently a reduction in the cellular water content. As already mentioned, exocytosis of renin appears to require granule swelling by intragranular water influx (Taugner et al. 1984; Skott and Taugner 1987); thus a calcium-activated chloride and water efflux from juxtaglomerular cells would impede granule swelling. As a result, renin secretion would appear to be inversely related to intracellular calcium concentration. cAMP, on the other hand, inhibits increases of intracellular calcium and thus inhibits the activation of the chloride channels and cell shrinkage. Consequently, cAMP would blunt the inhibition of renin secretion. The inhibitory role of cGMP on secretion could also be explained by this concept.

Patch clamp investigations on juxtaglomerular cells have revealed that cGMP increases the calcium sensitivity of the chloride currents (Kurtz and Penner, unpublished). This is compatible with the observation that cGMP requires normal calcium concentrations for its optimal effect.

Finally, regulation of renin secretion by PLA_2 is also conceivable. PLA_2 is located at the plasma membrane, and its activity is regulated by the subplasmalemmal concentration of calcium. As already mentioned, a possible inhibitory effect of PLA_2 activity on renin secretion could be mediated by lipoxygenase products.

Discovering whether one or even all three hypotheses on the inhibitory action of calcium on the exocytosis of renin is correct remains a task for future research.

5 Physiological Environment of Juxtaglomerular Cells

As mentioned in the introduction, during the past 20 years four basic mechanisms for controlling renin release from the kidney under normal physiological conditions have been described (Davis and Freeman 1976). These are:

1. An intrarenal baroreceptor
2. The macula densa receptor
3. The sympathetic nervous system
4. Hormones

In the following sections on attempt is made to discuss the possible cellular mechanisms by which these four mechanisms could exert control over renal renin release.

5.1 Baroreceptor

The existence of a baroreceptor which controls renin secretion has been inferred from the findings that the renal perfusion pressure significantly affects renal renin release (Davies and Freeman 1976; Keeton and Campbell 1981). An increase in perfusion pressure leads to a decrease in renin release, and a fall in perfusion pressure stimulates renin release.

The localization and mode of function of this baroreceptor have not yet been identified. Experimental observations and theoretical considerations led to the suggestion that the baroreceptor acts as a stretch receptor (Tobian 1960; Skinner et al. 1964; Fray 1980). If it is assumed that the baroreceptor is localized in the afferent arteriole with the juxtaglomerular cells, a search should be made for a mechanism by which stretch of the arteriolar wall could influence renin release from the juxtaglomerular cells. The idea that the juxtaglomerular cell itself could behave as a stretch receptor has recently been supported by the finding of Fray and Lush (1984) that isolated juxtaglomerular cells grown on elastic support respond to an increased stretch with a decreased rate of renin release. It has been pointed out that juxtaglomerular cells would be particulary suitable to act as mechanoreceptors because they are poor in contractile filaments and therefore are presumably neither involved in autoregulation nor in vasoconstriction (Taugner et al. 1984).

If stretch of the juxtaglomerular cell membrane does affect renin release, then the question arises as to how membrane stretch is translated into the intracellular regulation of renin secretion. The most convenient model for this signal transduction has been devised by Fray and coworkers, who suggest that stretch of the juxtaglomerular cell membrane influences renin secretion by altering the membrane potential of the cells (Fray and Lush 1984). This concept fits with a variety of results.

It has been found that smooth muscle cells respond to stretch with depolarization (Bühlbring 1955; Coburn 1987; Harder 1984; Harder et al. 1987). Since no difference in the electrical behavior between juxtaglomerular cells and smooth muscle cells has been observed so far, it could be inferred that juxtaglomerular cells also depolarize in response to stretch. Depolarization, however, has been found to be associated with inhibition of renin secretion (Churchill 1985). As already discussed, this inhibition of renin secretion by membrane depolarization could be mediated by voltage-activated calcium channels. Alternatively, stretch of ion channels could also directly increase the calcium permeability of the cell membrane (Kirber et al. 1988). It should be recalled that in isolated juxtaglomerular cells renin release is inversely related to the calcium conductance of the cell membrane (Kurtz et al. 1984).

The concept that the baroreceptor works by altering transmembrane calcium fluxes is supported by the observations that the inhibitory effect of pres-

sure on renin release is directly related to the extracellular concentration of calcium (Fray et al. 1983 a, b). In addition, calcium channel blockade prevents the inhibition of renin release by high pressure (Fray et al. 1983 a, b).

The concept presented still needs to be proved experimentally. A suitable method of investigation could be patch clamp studies using single juxtaglomerular cells located in isolated afferent arterioles, in combination with single cell calcium measurement. Then it should be possible to detect stretch-dependent changes in ion permeabilities and intracellular calcium concentrations.

Summing up, the mechanism of action of the baroreceptor has not been identified. Theoretically it could be explained by stretch-dependent alterations in the intracellular calcium activity of juxtaglomerular cells.

5.2 Macula Densa

The macula densa segment of the distal tubule is considered to have two important roles in renal function: first, to mediate a negative feedback mechanism between glomerular filtration and afferent arteriolar perfusion (tubuloglomerular feedback, TGF) and, secondly, to link renin release to dietary intake of sodium chloride (Briggs and Schnermann 1986).

It has been portulated that a receptor which "measures" the ionic composition of the tubular fluid in the late proximal tubule exists at the macula densa, of the distal tubule. In particular, the concentration of sodium chloride is an effective modulator of renin release from juxtaglomerular cells (Davis and Freeman 1976; Keeton and Campbell 1981); a high load of sodium chloride in the macula densa region suppresses renin release, whilst a low concentration stimulates renin release. The mechanism of signal transduction from the macula densa to the juxtaglomerular cells is still subject to discussion. There is growing evidence that mediation by TGF and the adaptation of renin release to sodium chloride intake by the macula densa of use the same signalling pathway (Briggs and Schnermann 1976).

Some evidence points towards the generation of a metabolic signal by the macula densa cells. Presently, it is thought that there are two ways by which macula densa cells could generate signals: either directly, by releasing a metabolite from energy metabolism, or indirectly by altering the sodium chloride concentration and the tonicity around juxtaglomerular cells.

The link between energy metabolism and the macula densa signal is considered initially. An increase in the load of sodium chloride to the early distal tubule leads to an enhanced rate of sodium chloride reabsorption. Since the reabsorption of sodium chloride is energy dependent, an increased load causes increased energy turnover in the tubular cells (Osswald et al. 1982), which in turn leads to an increased rate of hydrolysis of ATP, causing a rise

in the level of adenosine (Osswald et al. 1982). Adenosine, which is released from cells (Miller et al. 1978), is known to be a renal vasoconstrictor and a potent inhibitor of renin release (Spielman and Thompson 1982; Skott and Baumbach 1985). Therefore, the concept has been developed that adenosine is the macula densa signal that mediates the inhibition of renin release from juxtaglomerular cells caused by an increased load of sodium chloride in the macula densa region (Osswald 1984). Recently, it has been shown by in vitro experiments that the macula densa significantly influences the response of juxtaglomerular cells to adenosine (Itoh et al. 1985b).

If adenosine is the macula dense signal, the question arises of the mechanism by which it could inhibit renin release from the juxtaglomerular cells. Using incubated renal cortical slices it was shown that adenosine has a biphasic effect on the rate of renin release (Churchill and Churchill 1985). Low concentrations of adenosine inhibit renin release, with a nadir at 0.1 μM. At concentrations higher than 10 μM, adenosine was found to stimulate renin release from the slices. In a recent experiment using cultures of juxtaglomerular cells we also found an inhibitory effect of adenosine on renin release (Kurtz et al. 1988b). Since the stimulation of renin release was only observed with large, pharmacological doses of adenosine, studies should concentrate on the physiological concentration range of adenosine ($0.1-1$ μM), in which a significant inhibition of renin release was observed (Churchill and Churchill 1985; Itoh et al. 1985b; Kurtz et al. 1988b). This inhibitory effect appears to be mediated by adenosine A_1 receptors, since it can be mimicked by specific adenosine A_1 agonists such as cyclohexyladenosine (CHA) (Murray and Churchill 1984; Churchill and Churchill 1985; Kurtz et al. 1988b). Using isolated juxtaglomerular cells we found that 0.1 μM adenosine influenced neither the transmembrane calcium influx nor the intracellular calcium level, as monitored by the fluorescence signal of the calcium indicator dye quin 2. At this concentration adenosine had no detectable effect on the cAMP level (Kurtz et al. 1988b), or on the membrane potential (Bührle et al. 1986b); however, we noted a concentration-dependent increase in the level of cGMP (Kurtz et al. 1988b). Since there is evidence that a rise in the cytosolic concentration of cGMP inhibits the secretory process in the juxtaglomerular cells, it seems possible that adenosine inhibits renin release by raising cGMP levels.

Another method by which the macula densa could generate signals is conceivable. Recently it has been shown that renin release from isolated juxtaglomerular apparatuses is inversely related to the sodium chloride concentration at the corresponding macula densa (Skott and Briggs 1987). Increased sodium chloride concentration in the tubular fluid results in an increased furosemide-sensitive transtubular sodium chloride transport to the interstitial space between the macula densa and the juxtaglomerular cells (Gonzalez et al. 1988). Since this space is not well drained, the local sodium chloride concentration changes in relation to the tubular sodium chloride load (Gonzalez

et al. 1988; Persson et al. 1988). Juxtaglomerular cells have been found to behave as sensitive osmometers and an increase in extracellular tonicity causes inhibition of renin release and vice versa (Frederiksson et al. 1975; Skott 1988). However, this concept cannot yet explain the vasoconstricting effect of the macula densa signal.

5.3 Sympathetic Nervous System

It is well known that activation of the sympathetic nerves in the kidney leads to a rise in the rate of renin release (Davis and Freeman 1976; Keeton and Campbell 1981). The action of the sympathetic nervous system on renin secretion requires functioning β-adrenergic receptors (Taher et al. 1976), the existence of which has been demonstrated in the juxtaglomerular region (Atlas et al. 1977). Since β-adrenergic receptors are known to be coupled with adenylate cyclase in a stimulatory fashion, it can be inferred that the sympathetic nervous system stimulates renin release by raising the cellular cAMP level. In fact, in isolated rat renal juxtaglomerular cells, it has been shown that the β-adrenergic agonist isoproterenol raises intracellular cAMP (Kurtz et al. 1984). As discussed above, cAMP presumably enhances the rate of renin release either by a direct effect or by lowering the intracellular activity of calcium.

It may, at first, seem remarkable that stimulation of renal sympathetic nerves leads to stimulation of renal renin release. The dominant transmitter of the peripheral sympathetic nervous system is norepinephrine, which is known to be a calcium mobilizing hormone for various cells, acting via α_1-adrenoreceptors (Berridge and Irvine 1984). We have found that $10^{-5}\,M$ norepinephrine enhances transmembrane calcium influx and inhibits renin release from cultured juxtaglomerular cells (Kurtz et al. 1986a). $10^{-5}\,M$ norepinephrine was also found to inhibit renin release from renal cortical slices (Capponi and Valloton 1976) and isolated perfused kidneys (Logan and Chatzillias 1980; Opengorth and Zehr 1983). However, it has been shown that norepinephrine at concentrations lower than $10^{-5}\,M$ results in stimulation of renin release, which is mediated via β-receptors (Lopez et al. 1978). We may assume, therefore, that norepinephrine is released from sympathetic nerve terminals at concentrations which stimulate renin release via β-receptors.

5.4 Hormones

To date a number of hormones have been tested as to their effects on renal renin release. In this review we are only considering those hormones for which there is evidence that they act directly on juxtaglomerular cells. These hor-

Table 1. Hormones stimulating renin release

Hormone	Reference
β-Adrenergic agonists	Keeton and Campbell 1981
Dopamine	Kurtz et al. 1988d
Prostaglandin E_2	Keeton and Campbell 1981
Prostaglandin I_2	Keeton and Campbell 1981
Glucagon	Keeton and Campbell 1981
Parathyroid hormone	Keeton and Campbell 1981
Vasoactive intestinal peptide	Porter et al. 1983
Calcitonin gene-related peptide	Kurtz et al. 1988c

Table 2. Hormones inhibiting renin release

Hormone	Reference
Angiotensin II	Keeton and Campbell 1981
Arginine-vasopressin	Keeton and Campbell 1981
α-Adrenergic agonists	Keeton and Campbell 1981
Platelet-activating factor	Pfeilschifter et al. 1985
Neuropeptide Y	Hackenthal et al. 1987
Adenosine	P.C. Churchill and M.C. Churchill 1985
Atrial natriuretic peptide	Kurtz et al. 1986b

mones are classified according to their ability to either stimulate (see Table 1) or inhibit renin release (see Table 2).

Hormones which stimulate renin release such as β-adrenergic agonists, dopamine, prostaglandins E_2 and I_2 glucagon, parathyroid hormone, vasoactive intestinal peptide, and calcitonin gene-related peptide (CGRP) have been shown to activate adenylate cyclase in various cell types. It may be inferred, therefore, that these hormones also activate adenylate cyclase in juxtaglomerular cells, and consequently cause a rise in the cytosolic level of cAMP. In our studies we have demonstrated that isoproterenol, prostaglandin I_2, and CGRP elevate the intracellular concentrations of cAMP in isolated juxtaglomerular cells (Kurtz et al. 1984, 1988c). Since cAMP is a stimulatory signal for renin release it may be concluded that these hormones stimulate renin release from juxtaglomerular cells via a rise in cAMP induced by stimulation of adenylate cyclase.

The hormones listed in Table 2 which have been found to inhibit renin release can be separated into two classes. One of the classes is composed of hormones which are known to mobilize calcium and which presumably inhibit renin release by increasing the intracellular concentration of calcium. These hormones also activate PLC in various cells. This group of hormones includes

all vasoconstrictors, e.g., angiotensin II, arginine-vasopressin, norepinephrine, and platelet activating factor. We have shown that these hormones enhance the transmembrane calcium influx into the cells and lead to a rise in the cytosolic level of calcium (Kurtz et al. 1986a; Kurtz and Penner 1989).

Inhibition of the adenylate cyclase is considered as a possible mechanism by which neuropeptide Y (Hackenthal et al. 1987) and adenosine (Rossi et al. 1987) inhibit renin secretion. However, it is also conceivable that adenosine inhibits renin release by raising intracellular cGMP (Kurtz et al. 1988a). This also appears to be the pathway along which atrial natriuretic peptide inhibits renin secretion.

6 Conclusions

In this review, present knowledge of the cellular regulation of renin secretion from renal juxtaglomerular cells has been considered. It appears that calcium is the dominant intracellular regulator of renin secretion and that it acts by inhibiting exocytosis. How calcium exerts this effect is not yet clear, but contraction of myofilaments, opening of chloride channels, and activation of PLA_2 could be involved. C-kinase activation and cGMP seem to have an additional inhibitory effect on renin secretion, both in a calcium-dependent fashion. cAMP, on the other hand, stimulates secretion, presumably by decreasing intracellular calcium activity. GTP-binding proteins and electrical properties also seem to be involved in the control of renin secretion.

Present knowledge suggests that exocytosis in renal juxtaglomerular cells is regulated by mechanisms which differ from those of other secretory cells, where calcium and C kinase stimulate exocytosis. Revealing the reason for this unusual behavior remains a thrilling task for future research.

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Physiological Role of cGMP and cGMP-Dependent Protein Kinase in the Cardiovascular System*

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Contents

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* Dedicated to Professor Dr. H.-D. Söling, Göttingen (FRG), on the occasion of his sixtieth birthday.

Abbreviations

ANF Atrial natriuretic factor; *CAP* Catabolite gene activator protein; *cAMP-PK* cAMP-dependent protein kinase; *cGMP-PK* cGMP-dependent protein kinase; *EDRF* Endothelium-derived relaxing factor; *GC* Guanylate cyclase; *kDA* Kilodaltons; IP_3 Inositol-1,4,5-trisphosphate; *MLC* Myosin light chain; *MLCK* Myosin light chain kinase; *NO* Nitric oxide; *PAF* Platelet activating factor; *PDE* Phosphodiesterase; *PG-*I_2 Prostaglandin I_2 (prostacyclin); *PG-*E_1 Prostaglandin E_1; *PI* Phosphatidylinositol; *PLC* Phospholipase C; *PKC* Protein kinase C; *SDS-PAGE* Sodium dodecyl sulfate–polyacrylaminde gel electrophoresis; *SNP* Sodium nitroprusside; *VASP* Vasodilator-stimulated phosphoprotein; *SDS* Sodium dodecyl sulfate

1 Introduction

Cyclic 3′,5′-guanosine monophosphate (cGMP) was discovered in 1963 as one of the major nucleotides in urine (Ashman et al. 1963) 6 years after the first description of cyclic 3′,5′-adenosine monophosphate (cAMP) as a second messenger (Sutherland and Rall 1957). Initially the research on cellular regulation and function of cGMP was heavily influenced by the progress made in understanding the physiological roles of cAMP. This led to the discovery and initial characterization of enzymes which synthesize and degrade cGMP, and enzymes which are activated by cGMP (see Table 1). It was subsequently recognized that the cellular level of cGMP is regulated by a wide variety of hormones, drugs, and cellular conditions (Goldberg and Haddox 1977). Important findings were that cGMP is regulated by acetylcholine in the heart, by light in the retina, by various vasodilators in smooth muscle, and by the diarrhea-causing, heat-stable enterotoxin from *E. coli* in intestinal epithelia (see Table 1 for references).

After further progress in the enzymology of proteins important for cGMP metabolism and action, it became apparent that the regulation and possible functions of cGMP are quite distinct from those of other second messengers (for reviews see Walter 1984; Tremblay et al. 1988). In particular, the structure and regulation of guanylate cyclases and of GMP-dependent protein kinases (cGMP-PKs) are very different from those of their counterparts, i.e., adenyl-

Table 1. Major advances in the research of cGMP and cGMP-regulated protein phosphorylation

Year	Discovery	Selected references
1961/62	Initial characterization of cyclic nucleotide phosphodiesterases	Drummond and Perrott-Yee 1961; Butcher and Sutherland 1962
1963	Presence of cGMP in urine	Ashman et al. 1963
1969	Discovery and initial characterization of soluble and particulate guanyiate cyclases	Hardman and Sutherland 1969; White and Aurbach 1969; Schultz et al. 1969
1970	Demonstration of a cGMP-dependent protein kinase	Kuo and Greengard 1970
1970	First demonstration of a hormonal regulation of the cellular cGMP level	George et al. 1970
1971	Regulation of cAMP-hydrolysis by cGMP	Beavo et al. 1971
1973	Regulation of cGMP-hydrolysis by light in retina	Miki et al. 1973
1975/77	Several classes of vasodilators regulate the cellular cGMP-level	Diamond and Holmes 1975; Katsuki et al. 1977; Schultz et al. 1977; Arnold et al. 1977
1978	Regulation of intestinal cGMP levels by the heat-stable enterotoxin of *E. coli*	Field et al. 1978; Hughes et al. 1978
1980–1983	Discovery of endothelium-derived relaxing factor (EDRF) and demonstration that it regulates cGMP	Furchtgott and Zawadzki 1980; Holzmann 1982; Rapoport and Murad 1983
1981	Demonstration of the specific cellular localization of cGMP-dependent protein kinase in cerebellar Purkinje cells, smooth muscle cells and intestinal epithelial cells	Lohmann et al. 1981; Walter 1981; DeJonge 1981; Walter et al. 1981
1984/85	ANF regulates the cellular level of cGMP via activation of the particulate guanylate cyclase	Waldman et al. 1984; Tremblay et al. 1985
1985	Demonstration that cGMP regulates the retinal cation channel directly without involvement of a protein kinase	Fesenko et al. 1985; Koch and Kaupp 1985
1986	Identification of a cGMP-inhibited phosphodiesterase as the major target for new cardiotonic drugs	Harrison et al. 1986; Weishaar et al. 1986
1987	Demonstration that cGMP-dependent protein kinase participates in mediating vasodilator-regulated protein phosphorylation in human platelets	Waldmann et al. 1987
1987	Evidence that endothelium-derived relaxing factor (EDRF) is identical to nitric oxide (NO)	Palmer et al. 1987; Ignarro et al. 1987
1988	Demonstration of Ca^{2+}-dependent inhibition of retinal rod guanylate cyclase	Koch and Stryer 1988
1988/89	Cloning of *Drosophila*, bovine and human cGMP-dependent protein kinase isozymes	Foster et al. 1988; Kalderon and Rubin 1989; Wernet et al. 1989; Sandberg et al. 1989
1988/89	cGMP-dependent protein kinase lowers cytosolic calcium in smooth muscle	Felbel et al. 1988; Cornwell and Lincoln 1989
1989	Cloning of mammalian particulate guanylate cyclase and identification as ANF-receptor	Chinkers et al. 1989

ate cyclases and cAMP-dependent protein kinases (cAMP-PKs) (Murad et al. 1979; Lohmann and Walter 1984; Beebe and Corbin 1986; Tremblay et al. 1988). The diversity in structure and regulation of phosphodiesterase which hydrolyze cAMP, cGMP, or both cyclic nucleotides, has only recently been appreciated (Strada et al. 1984; Beavo 1988). In eucaryotes cAMP-PK apparently represent the major intracellular effector system for cAMP (Lohmann and Walter 1984; Beebe and Corbin 1986), whereas cGMP-PK does not mediate all intracellular effects of cGMP: specific cyclic nucleotide phosphodiesterases present in various tissues, and the light-inhibited cation channel of the retina are regulated directly by cGMP without the participation of protein kinases (see Table 1 for references).

Despite significant progress in understanding the regulation of cGMP synthesis and the enzymology of cGMP-regulated proteins, the physiological function of cGMP remained obscure until very recently. There is now significant evidence suggesting an important regulatory role for cGMP in the retina (Stryer 1986; Kaupp and Koch 1986) in cardiovascular cells such as smooth muscle cells and platelets (Ignarro and Kadowitz 1985; Walter et al. 1988), and in intestinal epithelia (DeJonge 1984).

This review does not attempt to examine all aspects of cGMP regulation and effects, but concentrates on the role of cGMP in the cardiovascular system, with particular emphasis on the functional significance of cGMP-dependent protein kinase and cGMP-regulated protein phosphorylation. Recent reviews concerning other aspects of cGMP research are available (Ignarro and Kadowitz 1985; Stryer 1986; Beavo 1988; Tremblay et al. 1988; Waldman and Murad 1988).

2 Regulation of the Cellular Level of cGMP: Guanylate Cyclases

2.1 Soluble Guanylate Cyclase

Following the initial discovery that acetylcholine elevates the level of cGMP in the heart (George et al. 1970), many hormones, neurotransmitters, drugs, and conditions were found to affect its level in a wide variety of tissues (Goldberg and Haddox 1977). In many cases the hypothesis was advanced that the intracellular effects of those substances increasing the level of cGMP are mediated by this cyclic nucleotide. Due to the complexities of enzymes involved in cGMP metabolism and the extensive "cross-talk" between different second messenger systems, many puzzling inconsistencies were observed in early studies, e.g., in smooth muscle agents promoting contraction and those causing relaxation both elevated the level of cGMP (reviewed by Goldberg and Haddox 1977). Later, several laboratories studying the enzymes which syn-

Table 2. Some properties of soluble and particulate guanylate cyclase (GC)[a]

Property	Soluble GC	Particulate GC[b]
Molecular weight (kDa):		
native	150	(150?)
with SDS	70+80	150
Subunit structure	Heterodimer	(Monomer?)
Other structural property	Heme-protein	Glycoprotein
Partial tissue distribution in mammals:		
Brain	+ + +	+
Heart	+ +	+
Liver	+ + +	+ + +
Lung	+ + +	+ + +
Vascular smooth muscle cells	+ +	+ + +
Endothelial cells	+ +	+ + +
Platelets	+ + +	−
Intestinal epithelia	−	+ + +
Activators	Nitric oxide (= EDRF)	ANF
	Nitrovasodilators	Heat-stable enterotoxin (intestinal epithelia)
	Protoporphyrin IX	
	Fatty acids	Resact (sea urchin sperm)

[a] Adapted from Radany et al. (1983), Kuno et al. (1986a,b), and Tremblay et al. (1988)
[b] The particulate guanylate cyclase from intestinal epithelia may be different from the ANF-stimulable particulate guanylate cyclase of other tissues

thesize cGMP provided important evidence demonstrating that cGMP is involved in smooth muscle relaxation. This work also led to the purification and characterization of two distinct forms of guanylate cyclases the soluble and particulate forms (Ignarro and Kadowitz 1985; Murad 1986; Tremblay et al. 1988).

Some properties of soluble and particulate guanylate cyclases are summarized in Table 2. Soluble guanylate cyclase is a heterodimer with two immunologically distinct subunits (Kamisaki et al. 1986) and is present in most mammalian tissues and cells, but is virtually absent in intestinal epithelia. The enzyme is activated by a variety of vasodilators which have been collectively called "nitrogen oxide-containing vasodilators" or "nitrovasodilators", substances containing or releasing the free radical, nitric oxide (Murad et al. 1978; Ignarro et al. 1981; Böhme et al. 1984). These agents include nitroglycerin, sodium nitroprusside (SNP), nitrites, nitrosamines, and hydroxylamines, which have been used as antianginal agents for more than a hundred years (Brunton 1867; Murrell 1879).

Early attempts to purify soluble guanylate cyclase demonstrated a gradual loss of SNP-induced enzyme activation during purification. Subsequent studies using new purification procedures established that soluble guanylate cyclase contains heme, which is essential for activation by NO-containing drugs, and may be lost with certain purification processes (Gerzer et al. 1981a; Gerzer et al. 1981b). This finding was confirmed by another group which additionally established that heme-free guanylate cyclase can be activated by protoporphyrin IX, a heme without the central iron, suggesting that NO-heme and protoporphyrin IX activate the enzyme to the same extent due to their similar steric configurations (Ignarro et al. 1984).

These results established soluble guanylate cyclase as the receptor for the NO-containing and NO-liberating pharmacological vasodilators, but it was not certain until very recently, whether such a mechanism is of physiological significance. However, there is now convincing evidence that the endothelium-derived relaxing factor (EDRF), first described in 1980 (Furchtgott and Zawadzki 1980), is identical with NO (Palmer et al. 1987; Ignarro et al. 1987). Endothelium-dependent vasodilators, i.e., acetylcholine, histamine, adenosine, bradykinin, serotonin, thrombin, and platelet activating factor (PAF) cause relaxation of vascular smooth muscle only in the presence of an intact endothelium, and may even cause smooth muscle contraction in the absence of endothelial cells. These endothelium-dependent vasodilators stimulate endothelial cells to produce EDRF, a labile substance distinct from prostacyclin. EDRF is responsible for smooth muscle relaxation and stimulates the production of cGMP (Holzmann 1982; Furchtgott 1984).

Two groups independently provided several lines of evidence that EDRF is nitric oxide (Palmer et al. 1987; Ignarro et al. 1987). The evidence included:

1. The demonstration that EDRF and nitric oxide have very similar, if not identical, chemical and biological properties, i.e., a biological half-life of 5–30 s, similar inhibitory effects on platelet aggregation and smooth muscle contraction, and inactivation by substances such as superoxide anions, hemoglobin, and methylene blue.
2. The demonstration that endothelial cells produce NO (measured by a chemiluminescence procedure or by binding to hemoglobin) in response to endothelium-dependent vasodilators (Palmer et al. 1987; Ignarro et al. 1987; Busse et al. 1987; Moncada et al. 1988). Endothelial L-arginine is the probable source of this NO (Palmer et al. 1988; Schmidt et al. 1988).

It is of interest that the excitatory neurotransmitter glutamate, which increases the level of cGMP in the cerebellum induces the release of a substance with strikingly similar properties to EDRF (Garthwaite et al. 1988).

All of these results indicate that soluble guanylate cyclase is the receptor for NO-containing or NO-producing vasodilators, and that NO itself is the endogenous nitrovasodilator. Certain pharmacological nitrovasodilators such as

organic nitrates (i.e., nitroglycerin, isosorbide dinitrate, isosorbide mononitrate) have to be enzymatically reduced to nitrites, which requires the presence of free sulfhydryl (SH) groups in the tissue. It is likely that depletion of these free SH groups in tissues is the molecular basis for the development of tolerance to the effects of organic nitrates, a phenomenon not observed with SNP which releases NO independently of SH groups (Ignarro and Kadowitz 1985).

A dithiol group is also critical for the activity of soluble guanylate cyclase because the enzyme is apparently activated when this dithiol group is oxidized (Tremblay et al. 1988).

Soluble guanylate cyclase can also be activated by unsaturated fatty acids and fatty acid peroxides (Glass et al. 1977; Graff et al. 1978). It has been demonstrated that platelet lysates release activators of soluble guanylate cyclase in a Ca^{2+}-dependent manner. These activators have been identified as arachidonic and linoleic acid (Gerzer et al. 1983; Gerzer et al. 1986). The findings may explain earlier observations that many substances which raise intracellular Ca^{2+} and activate Ca^{2+}-dependent pathways, e.g., acetylcholine, alpha 1-adrenergic and H_1-histaminergic agents, thrombin, also elevate the level of cGMP in many tissues. Whether fatty acids and their oxidized products are physiological activators of soluble guanylate cyclase, and whether the rather indirect Ca^{2+}-dependent activation of guanylate cyclase is part of a possible feed-back inhibition of Ca^{2+}-regulated pathways, remain to be established.

In certain systems Ca^{2+} may directly modulate guanylate cyclase activity. Cilia of the protozoans *Paramecium* and *Tetrahymena* contain a guanylate cyclase which is activated in a Ca^{2+}/calmodulin-dependent manner (Schultz and Klumpp 1984). However, Ca^{2+}/calmodulin-activated guanylate cyclases have so far not been detected in vertebrate tissues.

A recent finding of considerable interest is that guanylate cyclase present in bovine retinal rod outer segments is activated by decreasing Ca^{2+} concentrations, and that this activation requires the presence of a calcium-sensitive regulator protein (Koch and Stryer 1988). It is possible that this Ca^{2+}-dependent regulation of retinal guanylate cyclase represents the feed-back loop restoring the normal level of cGMP, which decreases after illumination. Whether retinal guanylate cyclase represents a distinct class of enzyme or whether it is related to either the soluble or particulate form has not been established.

An additional mechanism of regulating guanylate cyclase activity may be phosphorylation-dephosphorylation. The soluble guanylate cyclase from rat brain was found to be a substrate of protein kinase C in vitro (Zwiller et al. 1985), although the physiological significance of this finding is unclear.

2.2 Particulate Guanylate Cyclase

Particulate guanylate cyclase is present in most mammalian tissues as well as in many nonvertebrate cells (Waldman and Murad 1988; Tremblay et al. 1988). Guanylate cyclase is almost exclusively in particulate form in intestinal epithelia and renal glomeruli, whereas platelets do not contain a detectable level of this enzyme. Research on particulate guanylate cyclase was greatly stimulated by the discovery that atrial natriuretic factor (ANF) selectively activates the particulate form but not the soluble form (Waldman et al. 1984; Tremblay et al. 1985). ANF was originally discovered as a potent natriuretic and vasorelaxing agent present in extracts of atria (DeBold et al. 1981) and was subsequently purified, sequenced and cloned. Recent comprehensive reviews on the structure, diversity, and biology of ANF peptides are available (Laragh 1985; Needleman and Greenwald 1986; Genest and Cantin 1988). Although ANF, like many other hormones, may regulate more than one intracellular effector system, at least some of its effects appear to be mediated by the activation of particulate guanylate cyclase.

Cross-linking studies have identified two receptors for ANF which have molecular weights of about 66000 and 130000–150000. A subtype of the high molecular weight receptor correlates in its tissue and cellular distribution with particulate guanylate cyclase (Leitman et al. 1988), and copurifies with particulate guanylate cyclase from rat lung and adrenal gland (Kuno et al. 1986a; Paul et al. 1987). The 130000-dalton ANF receptor apparently contains two receptor species: one species may be converted to the 66000-dalton ANF receptor by reducing agents, whereas the second form is nonreducible. Apparently only the nonreducible ANF receptor, which represents only a minor fraction of all ANF receptors, is associated with particulate guanylate cyclase (Leitman et al. 1988; Pandey et al. 1988). The functions of ANF receptors not associated with particulate guanylate cyclase have not been established. Although ANF binds to purified preparations of particulate guanylate cyclase, this binding is not associated with increased enzymatic activity.

Interestingly, mammalian particulate guanylate cyclase has many biochemical properties in common with particulate guanylate cyclase from sea urchin sperm, e.g., they have similar molecular weights and they both have the properties of a glycoprotein (Radany et al. 1983; Kuno et al. 1986a).

Sea urchin sperm particulate guanylate cyclase, the receptor for the chemotactic peptide Resact (Shimomura et al. 1986), has been recently cloned (Singh et al. 1988). The deduced amino acid sequence predicts a transmembrane protein of 986 amino acids (calculated molecular mass of 106150 daltons) with a single transmembrane domain. Surprisingly, there is some homology between sea urchin guanylate cyclase and the catalytic domain of protein kinases, especially with members of the tyrosine kinase family. This may simply represent the homology of proteins with a nucleotide binding do-

main, or may classify guanylate cyclase as a protein kinase, and remains to be elucidated. However, the similarities of sea urchin sperm guanylate cyclase and mammalian particulate guanylate cyclase suggest that ANF-binding and guanylate cyclase activity are the properties of one protein. Most recently (Chinkers et al. 1989), successful cloning of the rat brain particulate guanylate cyclase clearly established that this enzyme is a membrane-spanning protein with an extracellular ANF-receptor domain and an intracellular guanylate cyclase catalytic domain, the latter of which has some homology with the recently cloned soluble guanylate cyclase (Koesling et al. 1988). In mammalian systems, the only established activator of particulate guanylate cyclase, in addition to ANF, is the *E. coli* heatstable enterotoxin (Field et al. 1978; Hughes et al. 1978; DeJonge 1984). The mechanism of activation of intestinal guanylate cyclase by this toxin appears to be different from the activation of particulate guanylate cyclase by ANF and Resact, since the intestinal guanylate cyclase and the receptor for the heat-stable enterotoxin are easily separated (Kuno et al. 1986b). It is therefore possible that intestinal guanylate cyclase represents a distinct species of particulate guanylate cyclase. No physiological activator of the intestinal enzyme is known. Future studies will address the question of whether guanylate cyclases represent a family of homologous proteins.

3 Regulation of the Cellular Level of cGMP: Phosphodiesterases

The cellular concentration of cGMP is not only controlled by synthesizing enzymes (guanylate cyclases) but also by degrading enzymes (phosphodiesterases). A striking example of primary regulation of cGMP concentration by phosphodiesterases is found in retinal rod outer segments. In this system, light activates a specific cGMP-phosphodiesterase resulting in reduced cGMP levels and closure of a cation channel (Stryer 1986; Kaupp and Koch 1986).

Although phosphodiesterases have been known for more than 20 years, their enormous complexity, diversity, and differential regulation prevented detailed understanding of them for many years. Improved purification and assay methods, together with the development of specific antibodies and selective inhibitors and activators, allowed the first general classification of phosphodiesterases present in mammalian tissues (Strada and Thompson 1984). There appear to be at least four major families of cyclic nucleotide phosphodiesterases:

1. Ca^{2+}-camodulin-stimulated phosphodiesterases capable of hydrolyzing both cAMP and cGMP

2. cGMP-stimulated phosphodiesterases which also hydrolyze cAMP and cGMP
3. cGMP-specific phosphodiesterases which selectively degrade cGMP
4. low-K_m phosphodiesterases which preferentially degrade cAMP

Some of the properties of these four families of phosphodiesterases are summarized in Table 3 which is based to a large degree on the information provided by a recent review (Beavo 1988). This article is the most recent and comprehensive summary of the rapidly expanding field of cyclic nucleotide phosphodiesterase. As stressed by Beavo, this classification and many experimental details concerning individual phosphodiesterases may have to be modified and expanded when more information on the structure and function of these enzymes at the molecular level is available. In order to understand the physiological role of phosphodiesterases at the cellular level it will be necessary to determine which of the various phosphodiesterases is present in a given cell type.

With respect to cGMP, a few general comments can be made:

1. Due to the cellular concentration of cGMP and the kinetic properties of the various phosphodiesterases, it appears likely that the low-K_m phosphodiesterases (type IV) do not contribute to the hydrolysis of cGMP.
2. An important family of phosphodiesterases for the regulation of cGMP levels is that of the Ca^{2+}/calmodulin-activated phosphodiesterases (type I). Elevation of intracellular Ca^{2+} activates this family and decreases the level of cyclic nucleotides, including that of cGMP. This has been demonstrated with cultured smooth muscle cells: agents elevating intracellular Ca^{2+} (i.e., angiotensin, vasopressin) inhibited the ANF- or SNP-induced rise of cGMP (Nambi et al. 1986; Smith and Lincoln 1987). This Ca^{2+}-dependent regulation of the level of cGMP is one more example of the extensive cross-talk between the various intracellular second messenger systems which may occur at every step of the activation cascade.
3. Phosphodiesterases are also regulated directly by cGMP through interaction of cGMP with a noncatalytic binding site, resulting in either activation (type II), inhibition (type IVb), or no apparent enzymatic change (type III) of such phosphodiesterases. This appears to be an important component of the mechanism of action of cGMP and is briefly discussed later (see Sect. 4.3).
4. Cyclic nucleotide phosphodiesterases are the targets of important drugs (Beavo 1988). Nonselective phosphodiesterase inhibitors such as isobutylmethylxanthine (IBMX), theophylline, and papaverine have been used for many years, both experimentally and clinically, as smooth muscle relaxants.

Some new cardiotonic agents (i.e., amrinone, milrinone, cilostamide) appear to be selective inhibitors of the cGMP-inhibited low-K_m phosphodi-

Table 3. Classification and properties of mammalian phosphodiesterases (PDEs)[a]

PDE family	Distribution	Molecular weight (kDa in SDS gels)	cAMP		cGMP		Selective inhibitors[b]
			K_m/μM	V_{max}/(μmol/min×mg)	K_m/μM	V_{max}/(μmol/min×mg)	
I Calmodulin-stimulated PDEs[c]	Most tissues	58–63 (native 120)	10–40	10–160	1–5	10–90	Calmodulin Antagonists
II cGMP-stimulated PDE[d]	Many tissues (i.e. heart, adrenal, liver, adipocytes, platelets)	105 (native 210)	36	120	11	120	?
III cGMP-specific PDEs[e]	a) Lung/platelet type	90 (native 180)	>10	?	0.3	?	Dipyridamole, M&B 22, 948
	b) Photoreceptor type	88+84+11 (native 180)	2000	500	60	1400	M&B 22, 948?
IV Low K_m-PDE[f]							
a) cAMP-specific[f]	Many tissues	60	2	95	310	33	Rolipram, RO 20-1724
b) cGMP-inhibited[g]	Heart, platelets, liver	110 (native 230)	0.2	6	0.1	0.6	cGMP, Milrinone, cilostamide
V Others (i.e., nonspecific PDEs)							

[a] Adapted from Beavo (1988)
[b] General *non*selective PDE inhibitors are IBMX (MIX), papaverine and theophyllin
[c] This group consists of several distinct subtypes. Some subtypes are phosphorylated by cAMP and/or Ca^{2+}/calmodulin-regulated protein kinases resulting in decreased affinity of the PDE for calmodulin
[d] Activated by cGMP in the concentration range of 0.1–10 μM
[e] PDEs with more than 50-fold selectivity towards cGMP as substrate
[f] May be stimulated by insulin in certain tissues
[g] Phosphorylated and activated by cAMP-dependent protein kinase; target for the new cardiotonic drugs such as milrinone, cilostamide, fenoximone

esterase (type IVb). The antidepressent rolipram selectively inhibits the cAMP-selective low-K_m phosphodiesterase (type IVa). Some of these drugs may however have other effects in addition to the inhibition of phosphodiesterases when used with intact cells or whole animals.

4 Possible Mechanisms of Action of cGMP

4.1 Activation of cGMP-Dependent Protein Kinases

In most eucaryotes cAMP is thought to achieve its physiological effects through the activation of cAMP-PKs, since regulatory subunits of cAMP-PKs are the only established intracellular cAMP-binding proteins and effector systems (Lohmann and Walter 1984; Beebe and Corbin 1986). One exception may be conductance in olfactory receptor cells, which is directly activated by both cGMP and cAMP (Nakamura and Gold 1987).

Following the discovery of a specific cGMP-PK in lobster tail muscle (Kuo and Greengard 1970), parallels were drawn to the well-described cAMP-PKs, and it was thought for many years that cGMP-regulated protein phosphorylation was the major if not the only mechanism of cGMP action in eucaryotes. There is now considerable evidence that cGMP-dependent protein kinase and

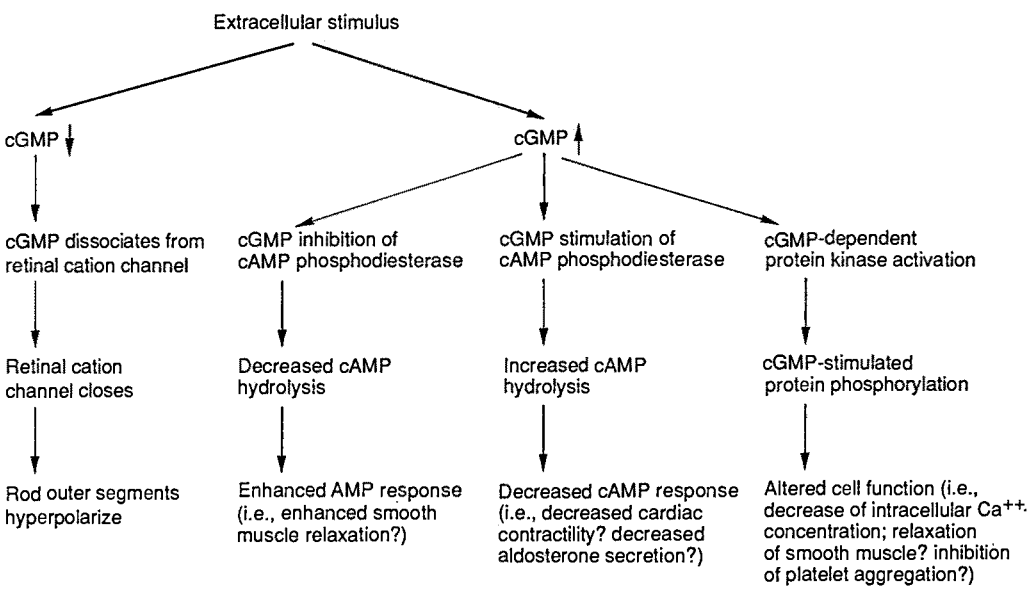

Fig. 1. Summary of established and postulated (?) mechanisms of action of cGMP in mammalian cells

cGMP-regulated protein phosphorylation are important for the regulation of specific cells such as smooth muscle cells, platelets, intestinal epithelia, and cerebellar Purkinje cells. This will be discussed in more detail later (see Sects. 5–7). However, increasing information on cGMP and cGMP-dependent protein phosphorylation has made it apparent that cGMP may achieve its diverse effects by more than one mechanism (Walter 1984). In addition to the activation of cGMP-PKs, cGMP directly regulates a cation channel in the retina and at least two classes of cyclic nucleotide phosphodiesterases as summarized in Fig. 1 (see also the following paragraphs).

4.2 Direct Regulation of the Retinal Cation Channel by cGMP

Retinal rod outer segments contain high levels of cGMP, which decrease in response to light (Miki et al. 1973), but no detectable level of protein kinases specifically regulated by cGMP (Walter 1984). There was a major breaktrough in cyclic nucleotide research in 1985 when two groups independently, and using different methods, discovered that cGMP directly regulates the retinal cation channel and keeps this channel open without the participation of a protein kinase (Fesenko et al. 1985; Koch and Kaupp 1985). It is possible that this type of cyclic nucleotide action is restricted to eucaryotic sensory cells.

4.3 Direct Regulation of Cyclic Nucleotide Phosphodiesterases by cGMP

Many years after the original discovery that cGMP stimulates the hydrolysis of cAMP in certain tissues (Beavo et al. 1971), several distinct types of phosphodiesterases were found to contain a noncatalytic binding site for cGMP. (For details of classification of characterization of phosphodiesterases see Table 3; Beavo 1988).

The observation that distinct families of cAMP phosphodiesterases exist is of considerable importance for understanding the mechanism of action of cGMP. These families are either inhibited or stimulated by physiological levels of cGMP. Regulation of cGMP could either increase or decrease the cellular level of cAMP. Hormones and drugs which increase cGMP could therefore have either synergistic or antagonistic effects with respect to cAMP-elevating hormones, depending on whether a cGMP-inhibited or cGMP-stimulated cAMP phosphodiesterase was present in a given cell type. A cGMP-stimulated phosphodiesterase may have an important physiological role in cardiac muscle cells which do not contain detectable concentrations of cGMP-PK (Joyce et al. 1984; Walter 1984).

It is well known that certain negative inotropic agents, such as acetylcholine, elevate the cGMP level in cardiac tissue (Goldberg and Haddox 1977). These agents could activate the cardiac cGMP-stimulated phosphodiesterase and lower the concentration of cAMP, thus causing a concomitant decrease in the positive inotropic effect of cAMP. Indeed, cGMP antagonizes the effect of cAMP in single heart cells, and there is considerable evidence suggesting that this cGMP effect is mediated by the cardiac cGMP-stimulated cAMP phosphodiesterase (Hartzell and Fischmeister 1986).

A cGMP-stimulated cAMP phosphodiesterase may also mediate the inhibitory effect of ANF on aldosterone secretion in the adrenal zona glomerulosa, which contains the ANF-stimulable particulate guanylate cyclase and a cGMP-stimulable cAMP phosphodiesterase (Beavo 1988; Tremblay et al. 1988). ANF could therefore activate cAMP hydrolysis and antagonize the cAMP-mediated secretion of aldosterone.

Whether inhibition of cAMP hydrolysis by cGMP is physiologically important is less certain. However, cGMP-induced inhibition of cAMP-hydrolysis could be important in vascular smooth muscle cells and platelets, since cAMP- and cGMP-elevating vasodilators are synergistic with respect to their inhibition of smooth muscle contraction and platelet aggregation (Walter et al. 1988). Furthermore, the presence of a cGMP-inhibited cAMP phosphodiesterase has been clearly demonstrated in human platelets (Macphee et al. 1986). Human platelets also contain a cGMP-PK which is believed to be important for the vasodilator-induced inhibition of platelet aggregation (Waldmann et al. 1987).

Future studies addressing the mechanism of action of cGMP have to establish, for each cell type, whether it contains hormone-regulated guanylate cyclases, cGMP-regulated phosphodiesterases, and cGMP-PKs with specific substrates. Experiments have to be designed to test the role of these different proteins in mediating the effects of those hormones and agents which elevate cGMP.

4.4 Other Possible Mechanisms of Action of cGMP

Established enzymes and proteins regulated by cGMP and their possible physiological functions are illustrated in Fig. 1. The regulation of cGMP could possibly have additional effects, e.g., it has been proposed that the flux of cyclic nucleotides leading to the production of protons, pyrophosphate, and free energy could be coupled to an unidentified cellular process (Goldberg et al. 1983). Further, very high levels of cellular cGMP could affect the activity of cAMP-PKs, but clear evidence for these mechanisms is lacking.

5 Structure, Properties, Cellular Distribution, and Regulation of cGMP-Dependent Protein Kinases

5.1 Structure and Properties

A protein kinase selectively activated by cGMP was first discovered in lobster tail muscle (Kuo and Greengard 1970) and subsequently in rat cerebellum (Hofmann and Sold 1972). The cGMP-PK was purified to homogeneity from bovine lung (Gill et al. 1976; Lincoln et al. 1977), extensively characterized by several laboratories, and the complete amino acid sequence was elucidated in 1984 (Takio et al. 1984). The biochemistry of purified bovine lung cGMP-PK has been reviewed in depth (Lincoln and Corbin 1983; Lohman and Walter 1984; Beebe and Corbin 1986; Edelmann et al. 1987) and will be only briefly discussed here.

The native holoenzyme (M_r 150000) is composed of two identical subunits with a molecular weight of 76, 331 (670 amino acids) as determined by sequence analysis. In contrast to cAMP-PKs, each monomer of cGMP-PK contains both cyclic-nucleotide binding capacity and catalytic activity, and these are not physically separated upon activation by cGMP. The cGMP-PK is therefore a "chimeric" enzyme containing both regulatory (cyclic-nucleotide binding) and catalytic domains which are apparently derived from two separate protein families (Takio et al. 1984). The regulatory domain of cGMP-PK displays high sequence homology with other cyclic nucleotide-binding proteins (i.e., regulatory subunits of cAMP-PK and catabolite gene activator protein (CAP) from *E. coli*) whereas the catalytic domain shows significant homology with other protein kinases (i.e., catalytic subunit of cAMP-PK, other serine/threonine protein kinases, and even tyrosine protein kinases) as illustrated in Fig. 2.

The recent demonstration that *Drosophila* genes code for putative cGMP-PKs composed of regulatory and catalytic domains within one protein, suggests that the divergence of cyclic nucleotide-dependent protein kinases and the appearance of specific cGMP-PKs is an early event in evolution (Foster et al. 1988; Kalderon and Rubin 1989). This is also supported by the recent observation that a specific cGMP-PK related to the mammalian enzyme is present in the unicellular eucaryote *Paramecium* (Miglietta and Nelson 1988). However, there is no evidence suggesting the presence of cGMP-PK in yeast. It will be interesting to compare the sequence of cGMP-PK with that of other cGMP-binding proteins, i.e., retinal cation channel and cGMP-regulated phosphodiesterases, when these sequences become available.

It is thought that the two monomers of cGMP-PK holoenzyme are arranged in an antiparallel manner such that the regulatory domain of one monomer inhibits the catalytic domain of the other monomer. This inhibition is released when cGMP binds to the enzyme. The extreme N-terminus of the

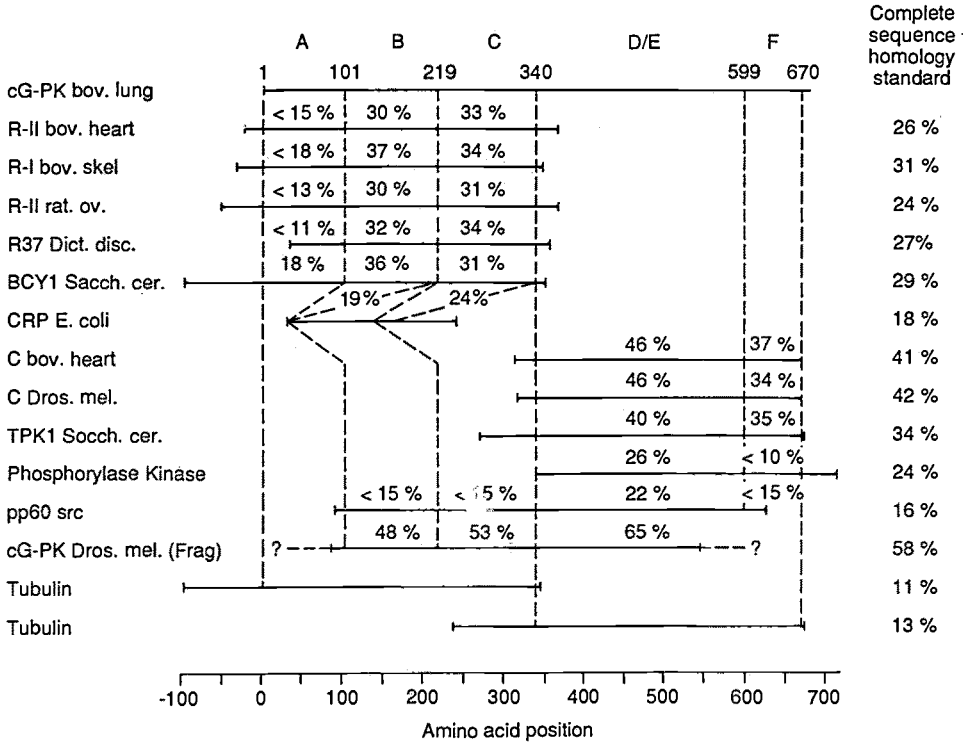

Fig. 2. Comparison of amino acid sequences of bovine lung cGMP-dependent protein kinase (cG-PK bovine lung) and related proteins. Published sequences of the proteins were obtained from the EMBL Data Bank and were compared to bovine lung cGMP-PK as standard using the complete sequence, the N-terminal domain **A** (amino acids 1–100, essential for dimerization and inhibition of kinase activity in the absence of cyclic nucleotides), domain **B** containing the cyclic nucleotide binding site 2 (amino acids 101–219), domain **C** containing the cyclic nucleotide binding site 1 (amino acids 220–340), domains **D** and **E**, containing the ATP binding site and kinase activity (amino acids 341–599), and the carboxy-terminal domain **F** (amino acids 600–670). Comparisons were made using the GAP Wisconsin program with the VAX computer of Würzburg University. The following proteins were compared: Various regulatory subunits of cAMP-PK (*R-II* from bovine heart, *R-I* from bovine skeletal muscle, *R-II* from rat ovary, *R37* from *Dictyosteliium discoideum*, yeast gene *BCY1* of Saccharomyces cerevisiae; cAMP receptor protein (*CRP*) from *E. coli*), catalytic subunits of cAMP-PK (*C*) from bovine heart and *Drosophila melanogaster* (*Dros. mel*); yeast gene *TPK 1* (of saccharomyces cerevisiae); phosphorylase kinase; the tyrosine protein kinase, *pp60 src*; and a fragment (Frag.) of a *Drosophila* gene coding for a putative cGMP-PK. Tubulin was included in this list as a control protein unrelated to the cGMP-PK. The homologies of the complete sequences and of the various domains are indicated in per centages

enzyme is important for inhibition of the catalytic domain in the absence of cGMP. Removal of the first 77 amino acids by treatment with trypsin generates a monomer enzyme with full activity in the absence of cGMP (Heil et al. 1987). Each monomer of cGMP-PK contains two cGMP-binding sites which can be distinguished by their different affinities for various cGMP-analogs, and which are responsible for the cooperative nature of cGMP binding and activation (Corbin et al. 1986; Heil et al. 1987). Half-maximal activation of cGMP-PK in vitro is usually observed with 20–100 n*M* cGMP.

The N-terminal region of the protein also contains the major sites for autophosphorylation, serines 50 and 72, and threonines 58 and 84. Considerably more phosphate is incorporated in the presence of cAMP. This type of autophosphorylation reduces the cAMP concentration required for half-maximal activation of the enzyme activity by about 10-fold without major effect on the cGMP-induced activation (Landgraf et al. 1986). It is presently unknown whether cGMP-PK activation by cAMP and autophosphorylation are of physiological significance, but it is possible that autophosphorylation of the enzyme in intact cells could make the enzyme available for activation by cAMP. It is interesting that an insect protein kinase related to mammalian cGMP-PK has been purified and shows similar affinities for both cAMP and cGMP (Vardanis 1980). Recent evidence suggests that bovine aorta contains two very similar but distinct forms of soluble cGMP-PK (Lincoln et al. 1988; Wolfe et al. 1989).

A distinct type of cGMP-PK (also called type II) has been characterized and purified from rat and pig intestinal brush border membranes (DeJonge 1981). The intestinal enzyme (M_r 84000 in denaturing conditions) is entirely membrane-bound and can be solubilized by detergents and by partial proteolysis. The latter process releases a 71000-dalton protein with cGMP-binding capacity and kinase activity. The intestinal enzyme is apparently linked to the membrane via a 15000-dalton fragment. Antibodies prepared against either lung or intestinal cGMP-PK demonstrate little or no cross-reactivity (DeJonge and Lohmann, unpublished). Autophosphorylation has also been demonstrated with the intestinal enzyme; one phosphorylation site is in the 15000-dalton membrane-anchoring fragment and another site is in the 71000-dalton fragment. Future studies will have to clarify whether additional forms of cGMP-PK exist in mammalian tissues and whether they compare with lung enzyme (type I) or intestinal enzyme (type II).

5.2 Cellular Distribution and Regulation

Using biochemical methods, i.e., measurements of cGMP-binding capacity, kinase activity, and photoaffinity labeling, cGMP-PK could only be demonstrated in certain tissues such as lung, heart, cerebellum, and smooth muscle

(Lincoln and Corbin 1983; Lohmann and Walter 1984). Development of specific polyclonal antibodies (Walter et al. 1980) allowed quantitative immunological as well as immunocytochemical studies with respect to the cellular and subcellular distribution of cGMP-PK. In a specific radioimmunoassay, cGMP-PK immunoreactivity was detectable in all rat tissues and cell lines tested, although high levels were found only in cerebellum, heart, lung and aorta (Walter 1981). The intestinal cGMP-PK (type II) has so far only been detected in intestinal brush border membranes (DeJonge 1981 and 1984; DeJonge and Lohmann, unpublished experiments).

Recently, high levels of cGMP-PK related to the bovine lung type (type I) were demonstrated to be present in rat and human platelets (Waldmann et al. 1986). Since platelets can be obtained as a homogenous cell population it could be estimated that the intracellular concentration of cGMP-PK is more than 1 μM (Nieberding and Walter, unpublished experiments), which is similar to the concentration of cAMP-PK in most mammalian cells (Hofmann et al. 1977; Lohmann and Walter 1984). Immunocytochemical studies clearly established that cGMP-PK is not ubiquitously distributed in mammalian cells as is cAMP-PK (Lohmann et al. 1981; Walter et al. 1981; DeCamilli et al. 1984); Joyce et al. 1984; Boyles et al. 1984; Joyce et al. 1986; Joyce et al. 1987). In mammalian tissues cGMP-PK has been detected in the following cell types:

1. Type I cGMP-PK (prototype in bovine lung)
   a) Specialized neurons such as cerebellar Purkinje cells and Purkinje cell-like neurons of the brain stem and dorsal cochlear nucleus (Lohmann et al. 1981; DeCamilli et al. 1984
   b) Vascular and somatic smooth muscle cells including pericytes, the less developed vascular smooth muscle cells of capillaries and small veins (Walter et al. 1981; Boyles et al. 1984; Joyce et al. 1984)
   c) Smooth muscle-like cells including myofibroblasts and myoepithelial cells, e.g., intraglomerular and extraglomerular mesangial cells of the kidney, Ito cells of the liver, interstitial cells of lung and kidney, certain capsule cells, and other fibroblasts of the myofibroblast type (Boyles et al. 1984; Joyce et al. 1984; Joyce et al. 1986; Joyce et al. 1987)
   d) Platelets (Waldmann et al. 1986)
2. Type II cGMP-PK (intestinal type)
   Brush border membranes of intestinal epithelia (DeJonge 1984)

So far it has been impossible to detect cGMP-PK in cardiac and skeletal muscle cells, hepatocytes, glial cells, most neurons except cerebellar Purkinje cells, connective tissue fibroblasts endothelial cells, white blood cells, macrophages, and secretory cells (Boyles et al. 1984; Joyce et al. 1984, 1986, 1987; Walter, unpublished experiments). However the possibility cannot be excluded that very low levels of cGMP-PK or a cGMP-PK immunologically distinct from the ones studied are present in some of these cell types.

Many biochemical studies and cytochemical results suggest that cGMP-PK (type I) is primarily a soluble enzyme. However, about 25% of the enzyme in vascular smooth muscle and more than 80% of the enzyme in platelets appears to be particulate with the characteristics of a peripheral membrane protein (Ives et al. 1980; Waldmann et al. 1986). A significant portion of platelet cGMP-PK may be cytoskeletal-associated (Nieberding and Walter, unpublished). As discussed previously, the intestinal cGMP-PK (type II) is entirely membrane-bound.

Of particular interest is the observation that the cGMP-PK of the heart is only detectable in vascular smooth muscle cells and pericytes but not in cardiac muscle cells. Although activation of cardiac cGMP-PK in response to acetylcholine has been observed (Lincoln and Keely 1981), the immunocytochemical results suggest that the negative-inotropic effects of acetylcholine in the heart are not mediated by cGMP-PK (Walter 1984), but may be mediated by cGMP-stimulated cAMP-phosphodiesterase (see Sects. 4.3, 4.4). However, the role of cGMP and of cGMP-regulated events in cardiac function remain controversial (Robinson-Steiner and Corbin 1986).

Another interesting example of the specialized cellular distribution of cGMP-PK is in the kidney. Here, cGMP-PK was detected primarily in contractile cells of the vasculature, including regular vascular smooth muscle cells and intraglomerular and extraglomerular mesangial cells, as demonstrated in Fig. 3. This distribution appears to correlate somewhat with the distribution of ANF-stimulable particulate guanylate cyclase (Tremblay et al. 1985; Ardaillou et al. 1986; Nonoguchi et al. 1987; Chabardés et al. 1987), and it is likely that ANF achieves some if not all its major diuretic and natriuretic effects through the activation of cGMP-PK in contractile renal cells.

In general, the presence of high levels of cGMP-PK in smooth muscle cells and contractile smooth muscle-like cells, including platelets, suggests an important regulatory role of cGMP-PK in these systems.

It is believed that cGMP-PK is primarily regulated by the intracellular level of cGMP and therefore by the actions of cGMP-elevating agents and hormones on intact cells. Although there are many technical difficulties in demonstrating the activation of cGMP-PK using intact cells (Fiscus and Murad 1988), activation of cGMP-PK and cGMP-regulated protein phosphorylation has been observed in response to ANF, EDRF, and nitrovasodilators in vascular smooth muscle (Rapoport et al. 1982, 1983; Fiscus et al. 1984, 1985), in response to acetylcholine in the heart (Lincoln and Keely 1981), and in response to SNP and cGMP-analogs in human platelets (Waldmann et al. 1987; Nieberding et al. 1987). This aspect of research is discussed in more detail in Sects. 6, 7.

It is possible that under certain conditions cGMP-PK can be activated by the intracellular level of cAMP (see Sect. 5.1), although at present there is no evidence for this in experiments using intact cells. The regulation of cGMP-

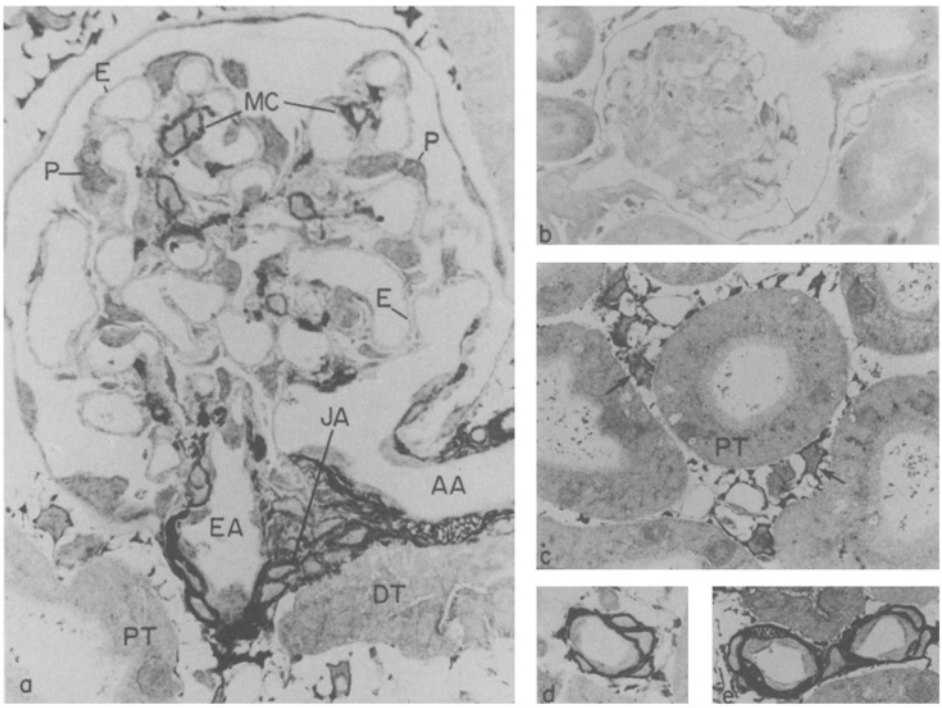

Fig. 3a–e. Light microscopic immunoperoxidase localization of cGMP-dependent protein kinase (*cGMP-PK*) in rat kidney cortex. In tissue sections incubated with immune serum (**a**, **c–e**), intense staining for cGMP-PK is visible in all contractile cells associated with the vasculature, while the corresponding cells in section incubated in preimmune serum (**b**) are consistently unreactive. Within the glomerulus (**a**) mesangial cells (*MC*) and the smooth muscle cells of the afferent (*AA*) and efferent arterioles (*EA*) are highly immunoreactive. In addition, extraglomerular mesangial cells within the juxtaglomerular apparatus (*JA* in **a**), the smooth muscle cells of the peritubular vasculature (**d**, **e**), and interstitial myofibroblasts (*arrows* in **c**) are highly reactive for cGMP-PK. Endothelial cells (**E**), podocytes (*P*), and the epithelial cells of the proximal (*PT*) and distal tubules (*DT*) are not significantly stained over background. Magnification: **a**×730; **b**×230; **c**×570; **d**×600; **e**×570 (From Joyce et al. 1986)

PK by a heat-stable modulator protein (Kuo et al. 1976) appeared to be due to the interaction of this modulator protein with the in vitro substrates and not due to the regulation of the kinase per se (Lincoln and Corbin 1983).

An area which has received relatively little attention is the regulation of the cellular level of cGMP-PK. In guinea pig lungs and heart the concentration of cGMP-PK was found to decline during development (Kuo 1975), whereas the emergence of cGMP-PK in rat cerebellum correlated with the differentiation of Purkinje cells (Bandle and Guidotti 1979). No change in the level of cGMP-PK was found during hormone-induced differentiation of neuroblastoma×glioma hybrid cells (Lohmann et al. 1983). Unfortunately, reports of nuclear cGMP-PK in liver and its increase following partial hepatectomy

(Linnala-Kankkunen and Mäenpää 1981; Tse et al. 1981) were not followed up with more rigorous methods. Preliminary reports indicate that the level of cGMP-PK in aorta and heart is affected by experimental hypertension (Ecker et al. 1988; Coquil et al. 1987). A recent interesting observation that the level of cGMP-PK in cultured vascular smooth muscle cells declined with higher passage numbers (Cornwell and Lincoln 1988) suggests that the expression of cGMP-PK is a property of differentiated vascular smooth muscle cells.

6 Established and Potential Substrates for cGMP-Dependent Protein Kinase

6.1 Criteria for the Functional Significance of Protein Phosphorylation

Several criteria have been proposed (Krebs and Beavo 1979) to establish that the phosphorylation of a given protein is an essential component for the regulation of cellular functions by hormones, factors and drugs. These criteria include the following points:

1. A protein kinase substrate thought to be involved in a cellular response should be stoichiometrically phosphorylated (or dephosphorylated) by the appropriate protein kinase (or phosphatase) at a reasonable rate both in vitro (cell extracts) and in vivo (intact cells).
2. The change in the phosphorylation of the substrate protein in vivo and in vitro should be accompanied by an appropriate change in the function of this protein.

Despite significant progress in the area of protein phosphorylation research, unequivocal satisfaction of these criteria has been possible for only a few of the thousands of protein kinase substrates which have been reported. With respect to cGMP-regulated systems, there has not been a single protein for which cGMP-regulated protein phosphorylation, correlated with an appropriate functional change, has been observed both in intact cells and broken cell systems. However, there are now a few candidate proteins whose phosphorylation by the cGMP-PK may be physiologically significant.

6.2 cGMP-Dependent Protein Kinase-Catalyzed Phosphorylation of Purified Proteins and Proteins in Broken Cell Systems

Studies using purified cAMP-PKs and cGMP-PKs demonstrated that these protein kinases have overlapping substrate specificity. However, in most cases (e.g., phosphorylation of glycogen synthase, pyruvate kinase, 1,6-fructose-bis-

phosphatase, triglyceride lipase, cardiac troponin I, myosin light chain kinase), the cGMP-PK was at least 10-fold less effective than the cAMP-PK, suggesting that phosphorylation of these proteins by cGMP-PK is physiologically irrelevant (Lincoln and Corbin 1983). In other cases (e.g., phosphorylation of histone H1, type I regulatory subunit of cAMP-PK, and phosphorylase kinase), cGMP-regulated protein phosphorylation was only observed with purified proteins but not in intact cells. Specific cGMP-regulated protein phosphorylation has been demonstrated in certain tissue extracts, and also in a few studies with intact cells, but the identity and function of these substrates is not known.

In vitro, the model peptide corresponding to the phosphorylation site in pyruvate kinase, i.e., Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), is a relatively inefficient substrate for the cGMP-PK by comparison with cAMP-PK. Nevertheless, Kemptide is useful for in vitro assays of cGMP-PK since it is commercially available, and methods of analyzing its phosphorylation have been standardized (Glass et al. 1981; Roskoski 1983).

Studies have established that histone H1 serine residues of the N-terminus, in particular serine 37, and serine residues of the carboxy-terminus are more selectively phosphorylated by cGMP-PK than by cAMP-PK, as defined by the ratio of V_{may}/K_m for each enzyme (Zeilig et al. 1981; Langan et al. 1981). In addition to histones, other chromatin proteins, such as the high mobility group protein HMG 14, are selectively phosphorylated by cGMP-PK in vitro (Walton et al. 1982; Palvimo et al. 1983). The presence of basic amino acids in the region of the phosphorylated serine or threonine appear to be critical for the activity of cGMP-PK. This has been concluded from studies with histones and by sequence analysis of the phosphorylation site in the type I regulatory subunit of cAMP-PK and the autophosphorylation site of cGMP-PK itself (Hashimoto et al. 1981; Glass and Smith 1983). However, as mentioned before, phosphorylation of nuclear proteins and the regulatory subunit R-I of cAMP-PK by cGMP-PK has neither been observed with intact cells nor is there any evidence of a functional relevance of these phosphorylations.

Another approach to the search for potential specific substrates for the cGMP-PK was the analysis of endogenous cGMP-regulated protein phosphorylation in crude fractions of various tissues. Not surprisingly, endogenous substrates effectively phosphorylated by the cGMP-PK have been detected primarily in those tissues containing high concentrations of cGMP-PK. These tissues include vascular and somatic smooth muscle (Casnellie and Greengard 1974; Casnellie et al. 1980; Ives et al. 1980), cerebellar Purkinje cells (Schlichter et al. 1978; Detre et al. 1984), intestinal brush border membranes (DeJonge and Lohmann 1985; DeJonge et al. 1986), and platelets (Waldmann et al. 1986).

The endogenous cerebellar substrate for cGMP-PK, a soluble protein of molecular weight 23000, has been purified to homogeneity, established as an

effective in vitro substrate of cGMP-PK, sequenced around the phosphorylation sites, and characterized as a potential inhibitor of phosphatases type I and type II a (Aswad and Greengard 1981a, b; Aitken et al. 1981; Nairn et al. 1985; Waalas and Greengard 1987). However, evidence for the regulation of this cerebellar protein, by phosphorylation in intact cells, has not been obtained so far, perhaps due to the lack of an appropriate cell culture model of cerebellar Purkinje cells.

Several membrane proteins of somatic and vascular smooth muscle – proteins designated GO (250000 daltons), G1 (130000 daltons), G2 (85000 daltons), and G3 (75000 daltons) – are specifically phosphorylated by an endogenous cGMP-PK (Walter et al. 1980; Casnellie et al. 1980; Ives et al. 1980). They may also be phosphorylated by cAMP-PK under certain conditions (Waldmann et al. 1986; Parks et al. 1987). In contrast to cGMP-PK which exists as a soluble and peripheral membrane-associated protein in smooth muscle, the most prominent substrate protein, G1, has ben characterized as a protein intrinsic to smooth muscle plasma membranes (Casnellie et al. 1980; Ives et al. 1980). This information, in combination with new information on plasmalemmal Ca^{2+}-pumps (Carafoli 1987), and a report that a plasmalemmal Ca^{2+}-pump of coronary smooth muscle is activated by cGMP and cGMP-PK (Popescu et al. 1985), led to considerable speculation that the plasmalemmal Ca^{2+}-ATPase of smooth muscle cells may be an important target for cGMP-elevating vasodilators and for cGMP-PK.

cGMP-dependent phosphorylation of a protein with molecular weight 135000 was observed when purified bovine lung cGMP-PK was added to purified bovine aortic plasma membrane Ca^{2+}-ATPase and incorporated into soybean phospholipid liposomes (Furukawa and Nakamura 1987). This study also revealed good correlation between cGMP-dependent phosphorylation of the 135000-dalton protein and the activation of Ca^{2+} uptake.

Another group also observed that cGMP-PK stimulated Ca^{2+}-ATPase activity in crude microsomal fractions from aortic smooth muscle cells, and that a 135000-dalton protein phosphorylated by cGMP-PK co-chromatographed with detergent-solubilized Ca^{2+}-ATPase through several steps (Rashatwar et al. 1987). More recently however, a report provided convincing evidence that plasmalemmal Ca^{2+}-ATPase and the major substrate (G1) of cGMP-PK in smooth muscle membranes are distinct entities (Baltensberger et al. 1988). This study also reported that cGMP-dependent thiophosphorylation failed to influence the Ca^{2+} uptake properties of sarcolemmal vesicles. Another group (Vrolix et al. 1988) was also unable to demonstrate direct phosphorylation of smooth muscle plasmalemmal Ca^{2+}-pumps by cGMP-PK, and showed that the cGMP-dependent phosphorylation of the Ca^{2+}-ATPase observed earlier (Furukawa and Nakamura 1987) may have been due to contaminating myosin light chain kinase. This study observed that cGMP-PK stimulated the activity of reconstituted Ca^{2+}-pumps from pig aorta and pig stomach smooth mus-

cle only in the presence of specific phospholipids such as phosphatidylinositol. The authors proposed that cGMP-PK may affect plasmalemmal Ca^{2+}-pumps indirectly via phosphorylation of an associated phospholipid (phosphatidylinositol) kinase.

There is reasonably good evidence that plasmalemmal Ca^{2+}-pumps are activated in intact vascular smooth muscle cells by cGMP-elevating vasodilators and cGMP-analogs (Furukawa et al. 1988), but it appears unlikely at present that this activation occurs via a direct phosphorylation of plasmalemmal Ca^{2+}-pumps by cGMP-PK. Clearly, more work with cell-free systems is required to elucidate the question of cGMP and cGMP-PK regulation of plasmalemmal Ca^{2+}-ATPase.

Another potential target for cGMP-PK is the myosin light chain kinase (MLCK) present in smooth muscle and contractile non-muscle cells (Sellers and Adelstein 1987), since MLCK from bovine tracheal smooth muscle and human platelets are in vitro substrates for both cAMP-PK and cGMP-PK (Nishikawa et al. 1984). Phosphorylation of MLCK by cAMP-PK which has also been observed with intact cells, reduces the affinity of MLCK for calmodulin, resulting in inhibition of its kinase activity (Nishikawa et al. 1984; Sellers and Adelstein 1987; Lamb et al. 1988). It is therefore likely that cAMP-dependent phosphorylation of MLCK is one mechanism by which cAMP-elevating substances modulate the contractility of smooth muscle cells and contractile non-muscle cells (see also Sect. 7). In contrast to the cAMP-system, cGMP-PK phosphorylation of MLCK has no effect on the latter's enzymatic activity and has not been observed with intact cells (Nishikawa et al. 1984; Sellers and Adelstein 1987). Phosphorylation of phospholambam by the cGMP-PK has been observed with isolated sarcoplasmic reticulum from cardiac and smooth muscle (Raeymakers et al. 1988) but similar results with intact cells are not available.

6.3 cGMP-Regulated Protein Phosphorylation in Intact Cells

There are very few studies which have reported cGMP-dependent protein phosphorylation in intact cells. In a study on intact rat aorta, SNP stimulated a pattern of protein phosphorylation which was mimicked by 8-bromo-cGMP, but only partially mimicked by cAMP-analogs or isoproterenol (Rapoport et al. 1982). The identical pattern of protein phosphorylation induced by SNP was also observed with rat aorta containing an intact endothelium in response to endothelium-dependent vasodilators such as acetylcholine (Rapoport et al. 1983; Rapoport and Murad 1983). Specifically, in intact rat aorta, nitrovasodilators and endothelium-dependent vasodilators increased the phosphorylation of two groups of proteins of apparent molecular weights 49000 and 24000–28000, respectively, and decreased the phosphorylation of

a 22000-dalton protein, later identified as myosin light chain (Rapoport et al. 1983; Draznin et al. 1986).

None of the proteins phosphorylated in response to nitrovasodilators in smooth muscle have been identified or studied in more detail with broken cell fractions. It is puzzling that the proteins phosphorylated in response to cGMP-elevating vasodilators in intact aorta are quite distinct from those phosphorylated by the cGMP-PK in smooth muscle membranes. Nevertheless, nitrovasodilator- and cGMP-induced decrease in the phosphorylation of myosin light chains in rat aorta (Rapoport et al. 1983; Draznin et al. 1986) may be an important component of cGMP-induced relaxation of smooth muscle (see also Sect. 7). Interestingly in another study using rat aorta, nitrovasodilators and cGMP-analogs not only inhibited the norepinephrine-induced contraction and phosphorylation of myosin light chains, but they also blocked norepinephrine-stimulated conversion of phosphorylase b to phosphorylase a, suggesting that the intracellular level of Ca^{2+} was reduced (Johnson and Lincoln 1985).

Human platelets are at present the only system in which cGMP-dependent protein phosphorylation has been shown to involve the same proteins both in intact cells and in cell extracts (Waldmann et al. 1986, 1987; Nieberding et al. 1987). In studies of intact human platelets, the cAMP-elevating vasodilator PG-E_1 stimulated the phosphorylation of several proteins with molecular weights of 240000, 68000, 50000, 24000 and 22000; this was mimicked by dibutyryl cAMP in intact platelets (not shown) and by cAMP in platelet membranes (Fig. 4a). 8-Bromo-cGMP and the cGMP-elevating vasodilator SNP increased the phosphorylation of a 50000-dalton protein in intact platelets, and this was mimicked by cGMP in platelet membranes (Fig. 4a). Consistently, cGMP-elevating vasodilators caused a small decrease in the phosphorylation of a 22000 dalton protein, whereas cAMP-elevating vasodilators increased the phosphorylation of this protein (Fig. 4a; Waldmann et al. 1987).

Use of specific inhibitors established that cAMP- and cGMP-stimulated protein phosphorylation in platelet membranes is mediated by cAMP-PK and cGMP-PK, respectively (Waldmann et al. 1987; Nieberding et al. 1987). A protein with an apparent molecular weight of 130000, phosphorylated in response to both cAMP and cGMP in platelet membranes, may be related to the G1-substrate of cGMP-PK in smooth muscle (Waldmann et al. 1986); however, regulation of this protein in intact platelets was not detectable (Waldmann et al. 1987).

A protein with an apparent molecular weight of 50000 is the only protein which has been shown to be phosphorylated both in response to cAMP- and cGMP-elevating vasodilators in intact platelets, and by cAMP-PK and cGMP-PK in platelet membranes. Limited proteolyses (Fig. 4b), tryptic fingerprinting, and phosphoamino acid analysis established that the 50000-dalton protein phosphorylated in intact platelets is identical to the protein of the same

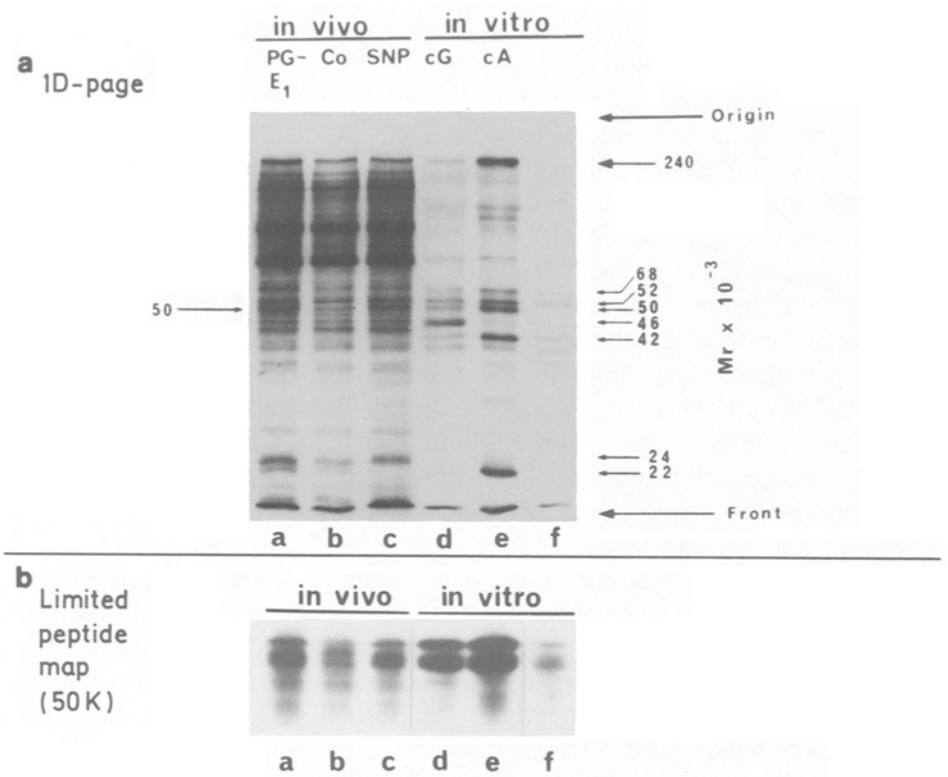

Fig. 4a, b. Autoradiograph showing the effects of PG-E_1, SNP and cyclic nucleotides on protein phosphorylation in platelets. The phosphorylation of intact platelets (in vivo) and of platelet membranes (in vitro) regulated by cyclic nucleotide-elevating agents (PG-E_1, SNP) and by cyclic nucleotides was analyzed by one dimensional (*1–D*) SDS-PAGE (**a**) and by limited peptide mapping (**b**). **a** The phosphorylation pattern induced by SNP or PG-E_1 in vivo (lanes *a–c*) and by cGMP, (*cG*) or cAMP (*cA*) in vitro (lanes *d-f*) was compared. In vivo, PG-E_1 stimulated the phosphorylation of at least four major proteins (M_r 240000, 68000, 50000, 24000) as indicated by *arrows*. The proteins were also phosphorylated in response to cAMP in vitro. SNP (in vivo) and cGMP (in vitro) enhanced the phosphorylation of one protein with M_r of 50000. In membranes, cGMP and cAMP also stimulated the phosphorylation of proteins with apparent M_r of 46000 and 42000 respectively. With intact platelets. PG-E_1 and SNP caused a moderate increase in the phosphorylation of two proteins with M_r of 52000 and 24000. **b** The 50000-dalton phosphoprotein used in this analysis was obtained from the phosphorylation experiments (shown in **a**) and digested by *Staphylococcus aureus* V8 protease. Limited digestion of the 50000-dalton protein produced a triplet of closely migrating phosphopeptides, which appeared to be identical for all conditions examined. Counting of the peptides obtained after V8 digestions indicated that SNP and PG-E_1 caused a two-to three fold increase in the phosphorylation of the 50000 dalton protein (From Waldmann et al. 1987)

molecular weight in platelet membranes, and also that it is closely related to a 46000-dalton protein, another specific substrate for cGMP-PK observed only in platelet membranes (Waldmann et al. 1987; Nieberding et al. 1987). This 50000-dalton protein, preliminarily designated as "vasodilator – stimulated phosphoprotein" (VASP), has recently been characterized in our laboratory as a peripheral membrane protein, and has been purified to homogeneity. Purified nonphosphorylated VASP migrates on SDS-PAGE as a 46000-dalton protein, is an excellent substrate for both cAMP-PK and cGMP-PK, and can be stoichiometrically phosphorylated (apparently at two distinct sites). Phosphorylated VASP migrates as a 50000-dalton protein on SDS-PAGE (Halbrügge and Walter 1989). The availability of VASP as a homogenous protein will allow new lines of approach for future investigation into its role in the mechanism of action of cyclic nucleotide-elevating vasodilators (see also Sect. 7). These investigations will include the study of the relationship of VASP to the following proteins:

1. A 49000-dalton protein present in aorta, which is phosphorylated in response to nitrovasodilators (Rapoport et al. 1983)
2. A 50000-dalton protein present in flounder intestinal brush border membranes, which is phosphorylated by both cGMP-PK and Ca^{2+}/calmodulin-dependent protein kinase (DeJonge et al. 1986)
3. A 50000-dalton substrate protein for the cGMP-PK present in purified preparations of smooth muscle plasmalemmal Ca^{2+}-ATPase (Vrolix et al. 1988)

Currently we are investigating whether VASP is involved in the regulation of phospholipase C (PLC) and other enzymes of phosphatidylinositol turnover (PI-turnover), and whether it participates in the the regulation of enzymes or proteins involved in Ca^{2+} removal or Ca^{2+} mobilization.

In intact platelets it has been demonstrated that cGMP-elevating vasodilators and cGMP-analogs stimulate both moderately and variably the phosphorylation of a 24000-dalton protein. This protein was more prominently phosphorylated in response to cAMP-elevating vasodilators (Fig. 4); the resulting 24000-dalton phosphoprotein has recently been identified as the β-subunit of glycoprotein Ib (Fox et al. 1987).

7 Role of cGMP-Dependent Protein Kinase and cGMP-Regulated Protein Phosphorylation in the Regulation of Vascular Smooth Muscle Cells and Platelets

7.1 Common Properties of Platelets and Vascular Smooth Muscle Cells

Vascular smooth muscle cells and platelets have emerged as important models for investigations into the roles of cGMP and cGMP-dependent protein

phosphorylation. Vascular smooth muscle cells and platelets have many properties in common, with respect to their regulation by cyclic nucleotides and cyclic nucleotide-regulated protein kinases (Walter et al. 1988):

1. Many hormones and substances activate both vascular smooth muscle contraction and platelet shape change, degranulation and aggregation. Those substances activating smooth muscle include noradrenaline, adrenaline, vasopressin, angiotensin, thromboxane, PAF, and endothelin; those activating platelet changes in clude thrombin, collagen, thromboxane, adenosine diphosphate PAF, vasopressin and adrenaline. Activation of these two cell types is mediated by an elevation of cytosolic Ca^{2+} and subsequent stimulation of Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) and protein kinase C (PKC) activity (see Sect. 7.2).
2. Activation of both vascular smooth muscle cells and platelets is inhibited by a variety of vasodilators and other substances which elevate either cAMP or cGMP. Vasodilators which elevate cAMP include β-adrenergic agents, prostacyclin (PG-I_2), PG-E_1, and forskolin; whereas nitrovasodilators, EDRF (nitric oxide), and ANF (the latter only in smooth muscle) represent the substances elevating cGMP (see also Sect. 2). The inhibition of smooth muscle and platelet activation by cyclic-nucleotide elevating vasodilators is mimicked by cell-membrane permeable analogs of cAMP and cGMP (Schultz et al. 1979; Haslam et al. 1980; Takai et al. 1982; Takai et al. 1984; Ignarro and Kadowitz 1985; Sellers and Adelstein 1987; Haslam 1987).
3. Both vascular smooth muscle cells and platelets contain high levels of cAMP-PKs and cGMP-PKs and specific substrates for each protein kinase. Activation of both cAMP-PK and cGMP-PK and subsequent protein phosphorylation has been observed with intact smooth muscle cells and platelets in response to appropriate hormonal or pharmacological stimuli (see Sects. 5, 6).

Current interest centers around the question of which steps of the activation cascade in vascular smooth muscle and platelets are inhibited by the cyclic nucleotides cAMP and cGMP and the subsequent protein phosphorylation which these cyclic nucleotides regulate. Before a discussion of the inhibitory roles of cyclic nucleotides it is necesary to consider briefly the activation cascade in these two cell types.

7.2 Mechanism of Smooth Muscle and Platelet Activation

Recent reviews on the mechanism of smooth muscle and platelet activation are available (Haslam 1987; Rasmussen et al. 1987), indicating that it is now generally accepted that cytosolic calcium is the second messenger for the acti-

vation of platelets and smooth muscle cells. The following steps are considered to be important for the activation process:

1. Elevation of intracellular calcium which may occur by receptor-dependent stimulation of PLC, or by receptor- or voltage-dependent stimulation of calcium channels (Meldolesi et al. 1987; Exton 1988). In the first case, activation of PLC increases the hydrolysis of phosphatidylinositol 4,5-biphosphate, generating 1,2-diacylglycerol and inositoltrisphosphate (Berridge 1987). 1,2-Diacylglycerol activates PKC whereas inositoltriphosphate releases Ca^{2+} from intracellular Ca^{2+} stores into the cytosol. At least two types of G-proteins (sensitive to either pertussis toxin or cholera toxin) appear to be involved in the regulation of PLC.

Alternatively, intracellular calcium may be elevated by voltage- and receptor-regulated Ca^{2+} channels in the plasma membrane, under the control of G-proteins or protein phosphorylation (Hofmann et al. 1987; Rosenthal and Schultz 1987). Thrombin-activated calcium channels have recently been demonstrated in platelet membranes (Zschauer et al. 1988), and it is noteworthy that thrombin-stimulated Ca^{2+} influx may occur before thrombin-induced Ca^{2+} release from internal stores (Sage and Rink 1987).

Future experiments will need to define the relative importance of these two distinct pathways, i.e., activation of PLC versus activation of plasmalemmal calcium channels, for the activation of smooth muscle cells, platelets, and other cell types.

It has also been proposed that the activation of platelets is due to PKC-mediated activation of Na^+/H^+ exchange with subsequent cellular alkalinization and Ca^{2+} mobilization (Siffert and Akkermann 1988). However, another study (Zavoico and Cragoe 1988) concluded that Ca^{2+} mobilization occurs independently of accelerated Na^+/H^+ exchange during activation of platelets.

2. Activator-stimulated increase in cytosolic Ca^{2+} and 1,2-diacylglycerol is followed by the *stimulation of Ca^{2+}/calmodulin-dependent MLCK and PKC* activity. Elevation of cytosolic calcium, primarily via plasmalemmal calcium channels, may only activate MLCK, although calcium is also capable of directly modulating the activity of phospholipases and PKC. It is firmly established that the phosphorylation of myosin light chains by MLCK is essential for the contraction of smooth muscle cells and platelets, whereas the role of PKC in this process is less well defined. Preferential activation of PKC also causes platelet aggregation and myosin light chain phosphorylation, although this phosphorylation is at sites distinct from the sites phosphorylated by the MLCK (Naka et al. 1983). The activation of PKC has been associated with both stimulation and inhibition phases of platelet aggregation (Haslam 1987). It seems possible that PKC has a stimulatory role in the early phase of platelet activation and an inhibitory role (negative feedback) in the later

phase of platelet aggregation. The latter effect could be due to a stimulation of calcium efflux (Pollock et al. 1987), or due to the phosphorylation of inhibitory G-protein (Crouch and Lapetina 1988).

Very similar mechanisms appear to operate during smooth muscle contraction. It was recently proposed that the initial phase of contraction was due to Ca^{2+}/calmodulin-dependent phosphorylation of myosin light chains, while the sustained phase (tonic phase) of smooth muscle contraction may be due to PKC-mediated phosphorylation of structural and regulatory components of the filamin-actin-desmin fibrillar domain (Rasmussen et al. 1987).

3. The process of inactivation of smooth muscle cells and platelets is due to the metabolism of the activating second messengers inositol-trisphosphate and 1,2-diacylglycerol (Berridge 1987), and due to the removal of the second messenger calcium from the cytosol (Carafoli 1987). In addition, proteins phosphorylated by MLCK and PKC during the activation phase have to be dephosphorylated in order to return to the basal state. High-affinity, low-capacity plasmalemmal Ca^{2+}-pumps, low-affinity, high-capacity Na^+/Ca^{2+}-antiporter, and high-affinity Ca^{2+}-pumps of the endoplasmic (sarcoplasmic) reticulum are all mechanisms physiologically important for the removal of calcium from the cytosol (Carafoli 1987; Exton 1988). The presence of both plasmalemmal and intracellular membrane Ca^{2+}-pumps is well established in smooth muscle (Carafoli 1987) and has recently also been demonstrated in human platelets (Enouf et al. 1988).

7.3 Inhibition of Platelet and Smooth Muscle Activation by Cyclic Nucleotide-Elevating Substances

The inhibitory effect of cyclic nucleotide-elevating vasodilators on both smooth muscle contraction and platelet aggregation has been known for many years, and current research activities in many laboratories are attempting to define the exact molecular site of this inhibitory effect (Walter et al. 1988; Lincoln 1989). At present it is impossible to offer a unified hypothesis without some oversimplification, because more than one pathway leading to activation or inactivation exists, and these pathways may differ in their relative importance in various types of smooth muscle cells and platelets. Furthermore, cGMP and cAMP may affect these activation and inactivation pathways at more than one site.

In the following paragraphs some of the possible mechanisms for regulation of smooth muscle cells and platelets by cyclic nucleotides and cyclic nucleotide-regulated protein phosphorylation will be considered.

7.3.1 Inhibition of Activation-Induced Protein Phosphorylation by Cyclic Nucleotides

It is well established that cyclic nucleotide-elevating substances and membrane-permeable analogs of both cAMP and cGMP inhibit the activation-associated phosphorylation of myosin light chains in both platelets and smooth muscle, and that they inhibit the PKC-mediated phosphorylation of an unknown soluble protein (M_r 47000) in platelets (Haslam et al. 1980; Takai et al. 1982, 1984; deLanerolle et al. 1984; Draznin et al. 1986; Waldmann and Walter 1989; Lamb et al. 1988).

The ability of cAMP-PK to phosphorylate MLCK (observed both in intact cells and in purified proteins), resulting in a decreased affinity of MLCK for calmodulin and thus inhibition of MLCK, strongly suggests that this is one mechanism by which cAMP-elevating agents inhibit activation of smooth muscle cells, platelets, and related contractile cells (Sellers and Adelstein 1987; Lamb et al. 1988). However, cAMP-PK is unable to inhibit the activity of MLCK already activated by calmodulin, whereas cAMP-elevating vasodilators are capable of reversing the activation of smooth muscle cells and platelets at a time when MLCK presumably already contains bound calmodulin. Therefore, it is very likely that cAMP inhibits the activation of smooth muscle cells and platelets at other sites in addition to the inhibition of MLCK (Sellers and Adelstein 1987; Waldmann and Walter 1989).

There is no data supporting the view that phosphorylation of MLCK by cGMP-PK is important for the action of cGMP-elevating vasodilators. Phosphorylation of MLCK by cGMP-PK has not been observed in intact cells, and in vitro-phosphorylation using purified proteins has no effect on the activity of MLCK (see Sect. 6). It has been proposed that cGMP-elevating vasodilators could stimulate a phosphoprotein phosphatase capable of dephosphorylating myosin light chains (Paglin et al. 1988), although at present there is little direct evidence for this mechanism.

Evidence that cAMP, cGMP, and their respective protein kinases may regulate other proteins of the smooth muscle contractile apparatus has been obtained using skinned tissue preparations (Pfitzer et al. 1982; Pfitzer et al. 1984), but the effects were quite small and have so far been undetectable in intact cells.

7.3.2 Inhibition of Activation-Induced Elevation of Cytosolic Calcium by Cyclic Nucleotides

The observation that cAMP- and cGMP-elevating substances inhibit the activation of both MLCK and PKC suggests that cyclic nucleotide-elevating vasodilators inhibit smooth muscle contraction and platelet aggregation at an early step of the activation cascade (Haslam et al. 1980; Takai et al. 1984,

Waldmann and Walter 1989). Indeed, cyclic nucleotide elevating substances and membrane-permeable analogs of cAMP and cGMP block or reduce activator-induced elevation of cytosolic calcium in both smooth muscle cells and platelets (Jones et al. 1984; Morgan and Morgan 1984; Kobayashi et al. 1985; MacIntyre et al. 1985; Hassid 1986; Nakashima et al. 1986; Blache et al. 1987; Kai et al. 1987; Cornwell and Lincoln 1988). Recently, evidence has been provided, using cultured tracheal and vascular smooth muscle cells, showing that cGMP-PK mediates the inhibitory effects of ANF and 8-bromo-cGMP on vasopressin- and depolarization-induced elevation of cytosolic calcium (Felbel et al. 1988; Cornwell and Lincoln 1989). Uptake of cGMP-PK into primary smooth muscle cells inhibited activation-associated elevation of calcium (using Fura-2 as indicator) – an effect blocked by antibodies against cGMP-PK. Further studies are necessary to establish which potential intracellular target of cGMP-PK (e.g. Ca^{2+} –ATPase, phospholipid metabolizing enzymes or ion channels are responsible for the calcium-lowering effects of cGMP. All of these results indicate that the regulation of calcium is an important action of cAMP- and, especially, cGMP-elevating substances.

7.3.3 Cyclic Nucleotide Regulation of Calcium Mobilizing Pathways

There is considerable evidence that both cAMP- and cGMP-elevating vasodilators block the agoinst-induced activation of the phosphatidylinositol cycle in human platelets. This inhibition decreases the level of inositoltriphosphate, resulting in diminished intracellular calcium and calcium/calmodulin-dependent protein phosphorylation, and reduces the level of 1,2-diacylglycerol and subsequent PKC-mediated protein phosphorylation (Haslam et al. 1980; Takai et al. 1982, Takai et al. 1984; Watson et al. 1984; Nakashima et al. 1986; Waldmann and Walter 1989). Although these studies with intact platelets indicate that the agonist-stimulable PLC activity could be an important target for both cAMP-PK and cGMP-PK, studies with broken cell systems except for a very recent preliminary report (Yada et al. 1989) have so far been unable to demonstrate a direct effect of cyclic nucleotide-regulated protein kinases on PLC itself (Haslam 1987; Waldmann and Walter 1989). Inhibition of agonist-induced phosphatidylinositol turnover by cGMP-elevating vasodilators has also been observed with vascular smooth muscle (Rapoport 1986).

Inhibition of agonist-stimulable PLC activity is certainly a potential mechanism by which cyclic nucleotide-elevating vasodilators block agonist-induced calcium mobilization. However, cyclic nucleotide-elevating substances are also capable of blocking the smooth muscle contraction and elevation of intracellular calcium induced by depolarization. Depolarization is believed to mobilize calcium by voltage-dependent calcium channels rather than via the PLC pathway (Cornwell and Lincoln 1989). In some smooth muscle cells

voltage-dependent calcium channels appear to be regulated by PKC (Exton 1988) and inhibited by cyclic nucleotides (Ousterhout et al. 1987). In snail neurons and cardiac muscle cells calcium currents are activated by injection of cGMP-PK or cAMP-PK, respectively (Paupardin-Tritsch et al. 1986; Osterrieder et al. 1982). However, it has not been established that plasmalemmal calcium channels of vascular smooth muscle cells and platelets are directly regulated by cyclic nucleotide-dependent protein kinases.

At present there is little direct evidence that the inositoltriphosphate-induced calcium release from intracellular vesicles into the cytosol is regulated by cAMP-PK or cGMP-PK (Haslam 1987; O'Rourke et al. 1989; Lincoln 1989) although a very recent study reported, that cAMP-dependent phosphorylation of a brain inositoltriphosphate receptor decreased its release of calcium (Supattapone et al. 1988).

7.3.4 Cyclic Nucleotide Regulation of Pathways Responsible for Calcium Removal

Cyclic nucleotides could affect intracellular calcium levels by regulating steps important for calcium removal from the cytoplasm. There is considerable evidence that treatment of intact vascular smooth muscle cells with cGMP-elevating agents and cGMP-analogs activates plasmalemmal Ca^{2+}-ATPase, causing enhanced calcium extrusion and decreased intracellular calcium (Kobayashi et al. 1985; Furukawa et al. 1988; Lincoln 1989). Activation of plasmalemmal Ca^{++}-ATPase in smooth muscle membranes by partially purified preparations of cGMP-PK has also been observed by some groups, although the precise role and mechanism of this activation at present is not clear (see also Sect. 6).

Substantial evidence indicates that platelets contain plasmalemmal Ca^{2+}-ATPase and Ca^{2+}-pumps associated with intracellular membranes (Enouf et al. 1988). Interestingly, calcium-accumulating vesicles in human platelets, which appear to be regulated by protein phosphorylation, contain a 23000-dalton membrane protein (22000-dalton in other studies) which is specifically phosphorylated by the catalytic subunit of cAMP-PK. However, the precise effect of cAMP-regulated protein phosphorylation on these calcium-accumulating vesicles is unclear, since both increased calcium uptake and calcium efflux have been reported (Käser-Glanzmann et al. 1979; Haslam et al. 1980; LePeuch et al. 1983; Enouf et al. 1985; O'Rourke et al. 1989). It has been tempting to speculate that the 23000-dalton protein phosphorylated by cAMP-PK is a regulatory protein of platelet Ca^{2+}-pumps (perhaps related to phospholamban of the cardiac sarcoplasmic reticulum) and an important component for the inhibitory effects of cAMP on platelet activation (Hettasch and LeBreton 1987; Adunyah and Dean 1987). However, the properties of this protein are quite distinct from those of phospholamban (LePeuch et

al. 1983). Also, the time course of phosphorylation in response to cAMP-elevating agents appears to be too slow to account for the inhibitory effect of platelet activation (Haslam et al. 1980; Waldmann, Nieberding and Walter, unpublished experiments). Data is not available concerning the effect of cGMP-PK on calcium uptake into platelet vesicles, and no consistent effects of cGMP-PK on calcium uptake into smooth muscle sarcoplasmic reticulum have been observed (Lincoln 1989).

Another important component of Ca^{2+} removal from the cytoplasm is the Na^+/Ca^{2+}-antiporter which may be a target for PKC in aortic smooth muscle (Vigne et al. 1988), but effects of cyclic nucleotide-regulated protein kinases at this site are not known.

7.3.5 Other Possible Functions of cGMP-Dependent Protein Phosphorylation

Activation of platelets is associated with a rapid reorganization of the cytoskeleton. The membrane skeleton participates in the cell shape-change response and the cytoplasmic actin filaments are involved in secretion, filopodia extension, and clot retraction (Fox 1987). It is therefore possible that cytoskeletal proteins are targets for both protein kinases involved in platelet activation and those involved in the inhibition of platelet activation. In this respect it is of interest that the β-subunit of glycoprotein Ib is phosphorylated by cAMP-PK in response to cAMP-elevating vasodilators (Fox et al. 1987). Glycoprotein Ib is essential for the adhesion of platelets to the subendothelium, contains binding sites for both thrombin and von-Willebrand factor on its extracellular domain, and has attachment sites for the membrane skeleton on its intracellular domain (Fox et al. 1987). Increased phosphorylation of a protein most likely representing the β-subunit of glycoprotein Ib has also been observed with cGMP-elevating vasodilators (Waldmann et al. 1987; Waldmann and Walter 1989; see also Fig. 4), although it has not been established that this is mediated by cGMP-PK. Nevertheless, reduced platelet adhesion has been noted in response to EDRF which can elevate cGMP (Sneddon and Vane 1988). It remains to be established whether functional properties of glycoprotein Ib are regulated by protein phosphorylation.

In vascular smooth muscle, cGMP-elevating vasodilators were observed to activate the Na^+, K^+,Cl^--cotransport system (O'Donnell and Owen 1986a, b), although it is unclear how this effect is linked to the inhibition of smooth muscle contraction. Considerable evidence suggests that cGMP and cGMP-PK inhibit the Na^+, K^+ Cl^--cotransport system of intestinal microvilli and activate an electrogenic Cl^--channel of intestinal crypt cells (De-Jonge and Lohmann 1985; O'Grady et al. 1988).

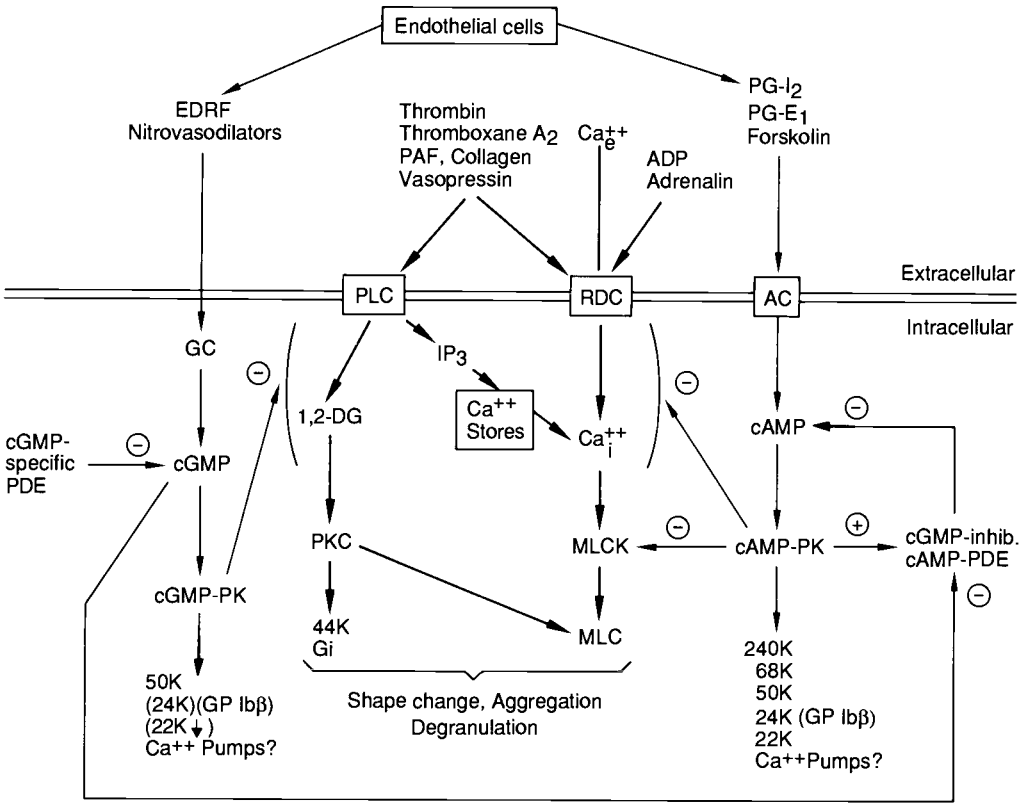

Fig. 5. Possible roles of cGMP and cGMP-PK in platelet function. *Arrows* indicate a stimulatory effect on the level of an extracellular or intracellular regulatory agent, stimulation of an enzyme, activation of a channel, or increased phosphorylation of a protein. Negative effects are indicated (⊖; ↓). Phosphorylation events are indicated by *heavy arrows* after the protein kinases shown. Abbreviations not previously defined are: $Ca^{2+}e$, extracellular calcium; $Ca^{2+}i$, intracellular calcium; *GC*, guanylate cyclase; *RDC*, receptor-dependent channel (calcium); *AC*, adenylate cyclase; *GPIb*, glycoprotein Ib; *cGI-cAMP-PDE*, cGMP-inhibited cAMP-phosphodiesterase; *50 K*, phosphoprotein with molecular weight of 50000 etc.; *Gi*, inhibitory G-protein

7.4 Current Model for the Regulation of Platelet Function by cGMP- and cGMP-Dependent Protein Kinase

Present knowledge of the regulation of platelets by second messenger regulated protein kinases, in particular by cGMP and cGMP-PK, is summarized in Fig. 5. Other sections of this review should be consulted for details of platelet and smooth muscle activation pathways (Sect. 7.2), function of guanylate cyclase (Sect. 2), and cyclic nucleotide phosphodiesterases (Sect. 3).

cAMP-elevating agents appear to inhibit platelet activation at the level of MLCK and/or steps regulating Ca^{2+} mobilization or Ca^{2+} removal. cAMP-

PK phosphorylates and activates the cGMP-inhibited cAMP-phosphodiesterase (PDE type IVa) which may constitute a mechanism of feedback inhibition for cAMP-elevating vasodilators. These substances may reduce the adhesion of platelets to the endothelium via cAMP-dependent phosphorylation of the β-subunit of glycoprotein Ib.

cGMP-elevating vasodilators activate cGMP-PK, producing increased phosphorylation of a 50000-dalton protein, slightly elevated phosphorylation of a 24000-dalton protein (most likely the β-subunit of glycoprotein Ib), and decreased phosphorylation of a 22000-dalton protein. As discussed earlier, both cAMP- and cGMP-dependent phosphorylation of the 50000-dalton protein correlate reasonably well with the observed inhibitory effects on platelet activation and activation-associated protein phosphorylation; this may respresent a possible site of synergism between cAMP- and cGMP-elevating vasodilators. An additional mechanism for this synergism may be cGMP-induced elevation of the level of cAMP by inhibition of the cGMP-inhibited cAMP-phosphodiesterase. cGMP-elevating agents appear to block platelet activation at an early step of the activation cascade regulating either Ca^{2+} mobilization or Ca^{2+} removal. Hydrolysis of cGMP is primarily catalyzed by a cGMP-specific phosphodiesterase which also contains a noncatalytic cGMP-binding site.

Since both cGMP- and cAMP-elevating substances inhibit platelet aggregation and activator-induced effects on calcium mobilization associated with increased phosphorylation of at least one protein (VASP), cAMP and cGMP may have certain intracellular effects in common. However, the regulation of cAMP and cGMP synthesis and hydrolysis and the pattern of cAMP- and cGMP-regulated protein phosphorylation clearly suggest that cAMP and cGMP also have separate intracellular effects.

8 Concluding Remarks

The last few years have yielded significant progress in the research areas dealing with cGMP- and cGMP-dependent protein phosphorylation. In retinal rods, an important regulatory role of cGMP in signal transduction of a light stimulus has been firmly established. It remains to be established whether channels directly regulated by cyclic nucleotides are also present in cell types other than sensory cells.

It is also firmly established that several important vasodilatory hormones and drugs use cGMP as their major intracellular second messenger. Elevation of cGMP can regulate specific phosphodiesterases by inhibiting or stimulating the hydrolysis of cAMP to produce either synergistic or antagonistic effects on cAMP-elevating hormones. cGMP-PK and cGMP-regulated protein

phosphorylation are not as widely distributed as the phosphorylation by cAMP-PK, but they appear to be of particular importance for the regulation of cerebellar Purkinje cells, smooth muscle cells, platelets, and intestinal epithelia.

There is substantial evidence that the regulation of cytosolic calcium level in vascular smooth muscle cells and platelets is an important, if not the most important, target for cGMP and cGMP-PK. Future studies have to clarify which steps of calcium mobilizing pathways (Ca^{2+} channels, phosphatidylinositol-turnover, inositoltriphosphate-induced Ca^{2+} release etc.) or calcium removal pathways (intracellular membrane and plasmalemmal Ca^{2+}-pumps, Na^+/Ca^{2+}-antiporter etc.) are regulated cGMP-dependent protein phosphorylation. It will be important to design useful experiments with broken cell systems for testing the direct role of cGMP-dependent protein phosphorylation on the regulation of cytosolic calcium. In addition, attempts should be made to identify, purify, and characterize functions of proteins for which specific cGMP-dependent phosphorylation, both in intact cells and in cell extracts, has been established.

Cell types such as vascular smooth muscle cells, platelets, and intestinal epithelial cells appear to be the best characterized systems to elucidate a physiological role of cGMP-regulated protein phosphorylation. Considerable evidence supports the view that cGMP-PK is an important effector component in the intracellular regulatory network of these cell types.

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Transferrin as a Muscle Trophic Factor

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1 Introduction

The questions of what regulates muscle cell growth and what maintains mature muscle fibers have long been attracting researchers. The role of nerves has been considered of great importance while that of humoral factors has been rather ignored. Phenomenologically the effects of nerves have been well studied; this is because the nerve is visible and easily accessible for operation, and because a great number of physiological observations document that muscle faithfully obeys the nerve's order. Thus Henneman (1980) entitled a chapter "Skeletal Muscle: The Servant of the Nervous System." Many observations concerning muscle atrophy may also have been important in the formation of this concept. For example, malformation of an animal which lacks a part of the spinal cord is accompanied by a lack of the muscle tissues which are normally innervated by motor neurons located at the defective region of the spinal cord (Weber 1851), and muscles atrophy following axon injury and in patients suffering from amyotrophic lateral sclerosis, a motor neuron disease.

Muscle fibers atrophy following loss of innervation. This is not explained solely by the immobilization effect of denervation. Acetylcholine (ACh) is not widely seen as transporting the nervous trophic stimulus; therefore, the presence of a neurogenic muscle trophic factor (or factors) – a substance(s) which prevents muscle from atrophying – is widely postulated (Fernandez and Donoso 1988). There have been repeated attempts to obtain such a trophic factor. Study of this substance is of practical importance since, if available, it could be used for treatment of certain neuromuscular diseases (Ochs 1988). Between 1979 and 1985, Oh and Malkelonis carried out substantial study on this problem. They obtained a protein from chicken sciatic nerve which they called sciatin and showed that it promotes the growth and differentiation of cultured muscle cells. Their work was broadly taken to indicate that sciatin is a neurogenic muscle trophic factor (Oh and Markelonis 1982b), and it profoundly impressed many researchers in their own and related fields.

During the same period, Ozawa and his coworkers isolated a myotrophic substance from chicken embryo extract, which had long been widely used as

an important component of culture medium (Carrel 1924), and showed it to be transferrin (Tf), a component of serum protein (Ii et al. 1981, 1982; Ozawa et al. 1983). They suggested that sciatin is Tf because of the similarity in their chemical and biological natures (Ii et al. 1981; Ozawa 1981). Oh and his coworkers then also concluded that sciatin is very similar to Tf or is Tf itself (Markelonis et al. 1982b; Oh and Markelonis 1982a; Oh et al. 1988).

Tf has long been used as an important component of serum-free culture medium (Neuman and Tytell 1961). Tf serves to transport iron to cells, and warm-blooded animal cells, such as muscle cells, require Tf for their proliferation and synthesis of Fe-containing molecules such as cytochromes and myoglobin. Therefore, Tf is undoubtedly essential for muscle cells. The problem is how Tf is provided to muscle cells: is it neurogenic or humoral? According to Oh et al. (1981) Tf (sciatin) is present in axoplasm. It is widely noted, however, that Tf is present in blood at very high concentration and is transferred into tissue fluid which immerses muscle cells.

Oh and his coworkers once considered Tf as a neurogenic trophic factor (Oh and Markelonis 1982b, 1984). Although they came to the conclusion that neurons do not synthesize Tf (Markelonis et al. 1985), they gave only an ambiguous answer to this problem in their recent review (Oh et al. 1988). In any case, the work of Oh's group is much too intriguing for many researchers of the trophic effect problem to abandon their concept.

In this review, we attempt to offer as clear an answer to this question as possible. For this purpose, muscle development, growth, and maintenance and the effect of denervation on muscle cells are first described. This is to show how cell proliferation and protein accumulation is essential for muscle cell growth and how innervation is necessary for muscle cell maintenance. General biochemistry and the cell biology of Tf is described in order to outline the nature of Tf and of Tf receptor (TfR). Thereupon, "transferrinology" in muscle cells is described. And finally, a detailed discussion considers whether Tf is provided to the muscle cells from the nerve or from the tissue fluid. Our conclusion is that Tf is a humoral factor furnishing Fe to muscle cells that is necessary for development, growth, and maintenance, although theoretically there are still some problems left to be clarified.

Here, it should be emphasized that both nervous stimulus and Tf are necessary for the growth and maintenance of muscle. Their roles seems to be distinct, however, since denervation causes growth arrest and atrophy in vivo in the presence of Tf. On the other hand, muscle cells in vitro absolutely require Tf. In its presence and in the absence of innervation, cultured myoblasts proliferate and fuse to form myotubes, which grow by accumulating muscle-specific proteins. In other words, myogenic cells not only proliferate but also differentiate under these conditions. The effect of Tf on cell growth is widely believed to be due to its promotional effect on cell division. In addition to this. Tf is required for protein synthesis and thus differentiation of the muscle cells.

The technical terms used in this review are defined, first. Myoblasts and muscle satellite cells are mononucleated cells which proliferate under favorable conditions and form polynucleated cells. Myotubes are polynucleated cells usually with central nuclei. Myoblasts and myotubes together are called myogenic cells. Mature muscle cells are called muscle fibers. Myogenic cells and muscle fibers together are called muscle cells. Muscle refers to tissue made of muscle cells and has an anatomical name, such as the long extensor muscle, of the toes. FeTf and apoTf are Tf with and without Fe binding, respectively. In situations with no potential for confusion, Tf is used in place of FeTf.

2 Cell Growth and Maintenance of Muscle Cells In Vivo and In Vitro

2.1 Cell Growth in Myogenesis

Muscle cell growth begins as soon as myogenic stem cells appear in embryos. Myogenic stem cells divide repeatedly, and this results in an increase in primordial muscle mass. After repeated proliferation, myoblasts, committed to final differentiation, fuse to form myotubes which do not proliferate further. These increase in size by fusing then with other myogenic cells and accumulating a large amount of muscle proteins such as myosin, troponin, and creatine kinase. Simultaneously, cross-striations, excitability of cell membrane, and contractility appear. Growth of multinucleated cells is usually remarkable immediately after birth, and then gradually declines. Finally, muscle cells cease growing to reach quasi-steady state. The cells are maintained at a certain level thereafter, although very slow growth may occur after reaching adulthood in certain animals. In this state, synthesis and degradation of protein must be at equilibrium.

2.1.1 Formation of Muscle Fiber

Almost all the skeletal muscles are derived from mesoderm (for review see Lewis 1910). Mesodermal mesenchyme first appears as amorphous cell masses between endoderm and exoderm and then forms many somites on both sides of the neural tube, which is located in the center of the embryo. Somites segregate into sclerotome, dermatome, and myotome. In chicken, trunk and limb muscles have been shown to originate from myotome (Christ et al. 1974; Chevallier et al. 1977; Kieny and Chevallier 1979), although Straus and Rawles (1953) reported that chicken abdominal muscles originate from the lateral plate. On day 2 of incubation – stage 13: about 19 somite (Hamberger and Hamilton 1951) – mesenchyme cells migrate from corresponding somites to the wing bud and proliferate in it (Jacob et al. 1978). The number

of migrating cells is estimated to be several hundred (Chevallier et al. 1977). In the hind led bud, mesenchyme cells begin to migrate as soon as corresponding somites appear (Jacob et al. 1978).

After migrating into the limb bud, the cells proliferate and condense at the proximal part to form mesenchymal basophilic compact cluster by stage 23 or 24 (Gould et al. 1972; Hilfer et al. 1973; Bennett et al. 1980). Demarcation of muscles begins to be clear at about stage 26, dorsal and ventral cell masses being separated. These cell masses then further separate into various muscles (McLachlan and Wolpert 1980). During these processes, myogenic stem cells greatly proliferate.

It is noteworthy in relation to the later context that at these stages nerve influence on proliferation may not be present (Shellswell 1977), and mesoderm cell proliferation is promoted by Tf and Fe in vitro (Sanders 1986; Sanders and Cheung 1988).

The cells composing prospective muscle masses appear as undifferentiated mononucleated ones, and after proliferation some of these mononucleated cells form polynucleated myotubes, called primary myotubes, leaving many myoblasts which are attached to the myotubes unfused. Myotube formation in vivo is due to the fusion of myoblasts (Mintz and Baker 1967). Primary myotubes in their first appearance are about 2 μm in diameter and much larger but variable in length. They have relatively pale cytoplasma and contain few myofibrils and some glycogen particles and perinuclear elongated mitochondria (Kelly and Zacks 1969a; McLachlan and Wolpert 1980; Stickland 1982; Ontell and Kozeka 1984; Ross et al. 1987a). Myosin and troponin T are detectable immunochistochemically (Miller et al. 1985; Miller and Stockdale 1987; Hagiwara et al. 1989b). Myoblasts do not have myofibrils but darkly stained nuclei, although one very early report stated that some myoblasts also have myofibrils (Godlewski 1902).

In time, primary myotubes increase in diameter as well as in length. These myotubes begin to be ensheathed together with myoblasts with basal membrane. Meanwhile, those myoblasts present on primary myotubes proliferate and finally fuse to form thin secondary myotubes leaving a few mononucleated cells unfused which are destined to be satellite cells (Ishikawa 1966).

In the rat fourth lumbrical muscle, primary myotubes begin to appear at embryonic day 16 and increase in number until embryonic day 17. The diameter of primary myotubes increases thereafter, however, their number does not increase (Ross et al. 1987a). In rat and mouse, innervation takes place soon after the formation of myotubes (Kelley and Zacks 1969b; Ontell and Kozeka 1984). Ross et al. (1987a) observed that there is an abundant nerve-muscle contact in primary myotubes, and that the axon terminal is functional. Secondary myotubes appear at embryonic day 19 and increase in number (Ross et al. 1987a). At first, primary myotubes can be discriminated from secondary myotubes by their size. Both primary and secondary myotubes first form

clusters intermingling with each other, then separating into small groups of myotubes showing a checkerboard pattern at cross-section preparation. Finally, the two types grow and can no longer the distinguished by their size.

Each single myotube together with a few mononucleated undifferentiated cells is ensheathed with basal membrane. These cells with poorly developed protoplasm are called satellite cells (Mauro 1961; Ishikawa 1966). With progressive development of myotubes, the nuclei move to locate at the subsarcolemmal position, and these muscle cells are then called muscle fiber. In rat newborn intercostal muscles, formation of muscle fibers is almost completed (Kelly and Zacks 1969a).

In summary, myogenic stem cells originating from myotome have at least three destinations. First, they fuse to form primary myotubes after proliferation; secondly, the myoblasts continue to proliferate and fuse to form secondary myotubes; and thirdly, they become satellite cells.

2.1.2 Growth of Muscle Fiber

The degree of growth and development of muscles at birth depends on the species of animal, and this greatly influence postnatal growth. For example, in the guokka, an Australian marsupial, the number of muscle fibers increases by about 30 times from the 10th to the 100th day after birth (Bridge and Allbrook 1970), whereas in mouse limb muscles the number hardly changes after birth (Rowe and Goldspink 1969).

In postnatal growth, the number of nuclei or amount at DNA contained in a muscle increases. Muscle fiber is said to contain about 100 nuclei per millimeter. The volume of cytoplasma which one single nucleus can rule is limited. Increase in the number of nuclei in a muscle fiber is therefore essential for the increase in its length. In chicken pectoralis and gastrocnemius muscles, the number of nuclei per muscle increases during the 128 days after hatching by about 50-fold and 25- to 30-fold, respectively (Moss 1968). Nuclear division was at one time considered to take place in muscle fiber (Enesco and Puddy 1964), but the view that nuclei in multinucleated muscle cells do not divide further is widely supported by observations on myogenic cell culture (Stockdale and Holtzer 1961; Yaffe and Feldman 1965). It is believed that the satellite cells proliferate and are taken into muscle fibers to increase the number of nuclei in a muscle fiber. When [^{3}H]thymidine is administered, muscle satellite cells take it up to synthesize DNA. Then mononucleated cells with radiolabeled nuclei gradually decrease in number, and labeled nuclei appear in muscle fibers (Moss and Leblond 1970, 1971).

The ratio of satellite cell nuclei to total muscle cells in mouse is high at birth (30%) but gradually decreases to approximately 5% at about 1 month (Cardasis and Cooper 1975; Schultz 1974). This decrease seems to be due to both a large increase in nuclei in a muscle fiber and a decrease in the absolute num-

ber of satellite cells (Schultz 1976). In the short flexor muscle of the toes in adult rat, for example, only one or two satellite cells are present per muscle fiber, which is 2–3 mm in length (Bischoff 1986a). Satellite cells of muscles of about 1-week-old animals are rich in cytoplasm, which contains numerous free ribosome and well-developed Golgi apparatus, mitochondria, microfilament, and microtubes, and they look active. In contrast, these cells in 1- to 10-month-old animals have poorly developed cytoplasmic organella and condensed nuclear chromatin (Schultz 1976). In adult muscle, satellite cells are thus called "dormant."

Muscle fiber also grows by increase in the cross-sectioned area. In mouse biceps muscle of the arm, a single muscle fiber contains about 75 myofibrils at birth. The number increases in approximately linear-correlation with the area. In adult mouse, a large fiber contain about 1000 myofibrils (Goldspink 1970). During the growth from newborn to adult, the number of myofibrils increases 10- to 15-fold. The increase in number has been suggested to be due to longitudinal splitting of the myofibrils (Goldspink 1970, 1971, 1980). When the length of muscle fiber increases, the length of myofibrils increases. This suggests the addition of new sarcomeres to myofibrils. The site of addition is suggested to be near the myotendonous junction (Goldspink 1970; Griffin et al. 1971).

Multinucleated muscle cells grow by accumulating muscle-specific protein. Proteins such as myosin, actin, and others accumulate (Herrmann 1952; Ohshima et al. 1965) and compose myofibrils (Fischman 1972). Soluble proteins such as creatine kinase, other enzymes (Eppenberger et al 1962/63; Reporter et al. 1963), and myoglobin (Low and Rich 1973; Kagan and Freedman 1973) are also collected. If the accumulated amount of protein per nucleus were constant, it would be the number of nuclei that actually determines muscle cell growth; in other words, growth might be entirely dependent on proliferation of myoblast or satellite cell. However, the amount of protein per nucleus increases with growth. In chicken, the ratio of the number of nuclei to muscle wet weight increases with two-thirds power to age during the 1st month of life (Moss et al. 1964). The ratio of wet weight (ng) per nucleus is 0.9 at birth and 6.3 at day 266 in pectoral muscle and 1.33 at birth and 5.3 at day 266 in gastrocnemius muscle (Moss 1968). Considering that protein content in cellular materials is about 10% (Giese 1979), it is clear that the protein/nucleus ratio increases with age. In human youth, the ratio of muscle protein to DNA increases linearly with age up to about 10 years in boys and about 17 years in girls. In boys the ratio increases with the power of 3 to age after 10 years (Cheek and Hill 1970; Cheek 1985).

During the stages of development and growth, the amount of protein synthesized may exceed the amount degraded. Finally, the amounts of synthesis and degradation become equilibrated at the adult stage. To maintain the differentiated state well-balanced protein turnover is required (Millward 1980; Obinate et al. 1981).

In summary, muscle cells grow not only by proliferation of mononucleated cells but also by protein accumulation in polynucleated cells. When muscle cells mature, protein turnover should be well balanced to maintain the steady state. From this standpoint, cell proliferation and protein accumulation and protein turnover can be taken as good markers in a study of muscle cell growth and maintenance, respectively.

2.1.3 In Vitro Growth of Myogenic Cells

Myogenic cell culture is widely used to study muscle cell growth, especially in studies on growth-promoting factors. Myoblasts present after the formation of secondary myotubes are the usual starting materials. Myoblasts present prior to or at the time of appearance of primary myotubes (Bonner and Hauschka 1974; Hauschka et al. 1977; Miller et al. 1985; Miller and Stockdale 1987) and satellite cells (Lewis 1915; Konigsberg et al. 1975; Kagawa et al. 1977, 1978) are used for specified purposes. Satellite cells on a muscle fiber with intact basement membrane can also be cultured (Bischoff 1986a, b).

These mononucleated myogenic cells multiply and finally form myotubes in appropriate culture medium in the absence of nerve cells, but suitable Tf or its equivalent is required for these processes. A single myoblast in culture separated from other cells proliferates (Konigsberg 1963), and proliferated myoblasts then fuse to form myotubes (Cooper and Konigsberg 1961; Stockdale and Holtzer 1961). In chicken myoblasts in young culture, the time required for one cell cycle is about 9.5 h (Holtzer and Bischoff 1970). After repeated proliferation, prolongation of G_1 occurs, myoblasts are committed for final differentiation, and they begin to synthesize muscle-specific proteins (Okazaki and Holtzer 1965; Shainberg et al. 1970; Paterson and Strohman 1972; Chi et al. 1975; Nadal-Ginard 1978; Devlin and Emerson 1979; Nguyen et al. 1983). Finally, myoblasts fuse to form myotubes (Cooper and Konigsberg 1961; Okazaki and Holtzer 1966). After fusion, myotubes accumulate proteins and also fuse with other myogenic cells, thereby increasing in size (growth of myotubes). During these processes, myogenic cells take on various muscular functions (Lewis 1915; Hagiwara et al. 1987). The development of myoblasts to functional myotubes takes place without the motor nerve, but requires Tf or Fe (Ozawa et al. 1983). It is generally believed that cultured myogenic cells do not differentiate beyond the myotube stage into the muscle fiber stage. Many attempts have been made to break through this limitation, but none seems to have succeeded. This limitation is an important factor in research on muscle cell growth when cultured myogenic cells are adopted for experimental models.

2.2 Regulation of Growth and Maintenance of Muscle Cells

Muscle cells grow and are maintained under the influence of nerve and tissue fluid; influences of other cells and matrices which maintain contact with these cells are beyond the scope of this review. Here, the focus is on nervous trophic effect on muscle cells. The important question is whether the trophic effect is mediated by ACh, or whether a specific trophic factor for muscle cells other than ACh is released from the nerve terminal.

2.2.1 Humoral Factors

The effects of various humoral factors on muscle cells are very important but, except Tf, not detailed in this review (see Florini 1987). It is enough to emphasize that muscle cells can take up many substances from tissue fluid, especially when specific receptors are present, and when these substances are provided in sufficient quantity from circulating blood to the tissue fluid.

2.2.2 Denervation and Maintenance

Muscle cells are innervated by motor neurons. An axon from a motor neuron ramifies after it penetrates into a muscle and innervates many muscle fibers (for review see Burke 1981). A single muscle fiber it is innervated by several axons from different motor neurons at the initial stage, and upon maturation it is innervated by a single motor neuron (Redfern et al. 1970; Dennis et al. 1981; for review: Bennett 1983). There is no doubt that a single neuron has a trophic effect on the group of muscle fibers which it innervates.

Dissection of the upper lumbar medulla and dorsal roots which prevents the activation of motor neurons results in muscle atrophy. However, electrical stimulation of the motor fibers can prevent the muscle from atrophying (Eccles 1941). It is noteworthy that the effects of such stimulation are the same whether administered for 2 h or 24 h per day.

Denervation is frequently used to examine the nervous trophic effect on muscle cells. Following denervation, muscles atrophy and this quickly progresses; muscle wet weight decreases by 70% – 80% within a month, and the cross-section area of muscle fibers is reduced. Atrophy progresses slowly thereafter, but muscles can be identified even 1 year later by their color and texture (Sunderland and Ray 1950; Engel and Stonnington 1974). Histologically, cross-striations are well preserved, although the diameter of the fiber and the volume of sarcoplasma are reduced. The essential feature of the change is not degeneration but atrophy, although vacuoles are sometimes observed. Some investigators found a decrease in the number of mitochondria at the time of initial change (Miledi and Slater 1968; Gauthier and Dunn 1973), and other found in increase (Bajusz 1964). This discrepancy may

be due to difference in the time of observation (Engel and Stonnington 1974). Since red muscle fibers with many mitochondria are more strongly affected than white muscle fibers following denervation (Tomanek and Lund 1973; Karpati and Engel 1968a, b; Engel and Karpati 1968; Niederle and Mayr 1978), a relative decrease in mitochondria in a muscle may be possible.

Denervation results in an increase in glycogen and DNA contents (Gutmann 1962, 1964). The DNA increase may be due to an increase in satellite cells (Hess and Rosner 1970). Muscle atrophy is probably the results of an unbalanced protein metabolism. There have been controversial findings on protein synthesis: it may decrease (Goldberg 1969) or increase (Pater and Kohn 1967); in other cases, it may first decrease and then increase (Goldspink 1976, 1978). These discrepancies may be caused by the difficulty in obtaining accurate kinetic data on protein metabolism (Zak et al. 1979).

In denervated muscle, calcium-activated neutral proteinase activity increases, suggesting greater protein degradation (Elce et al. 1983; Hussain et al. 1987). Calcium-activated neutral proteinase is present as soluble enzyme of cytoplasm and is not enclosed in organella as is lysosome (Suzuki et al. 1987).

2.2.3 Development and Growth of Muscle Cells Without Nerve

Myotubes are formed without innervation. This is supported by experiments on myogenic cell culture (Konigsberg 1963) or transplantation of pieces of primordial muscle mass (Muchmore 1957, 1958). Muscle cells grow after birth without innervation. When the sciatic nerve of newborn rat is cut, the soleus and the extensor longus muscle of the toes grow for at least 14 days. Increase in wet weight of these denervated muscles is 40.5% and 64.2% of the increase in respective control muscles. The diameter of muscle fibers increases for 14 days, then decreases, and by 60 days has become thinner than that in newborn rat (Zelena 1962). The amount of increase in dry weight of denervated muscle is half that of the innervated control after neonatal denervation (Schapira and Drayfus 1950, cited in Zelena 1962). In newborn rat muscle, a single muscle is innervated by multiple nerves (Redfern et al. 1970). At 9 days, a population of muscle fibers innervated by a single motor nerve appears and then increases. At 15 days, multiple innervation finally disappears (Miyata and Yoshioka 1980). The coincidence of the periods of growth of denervated muscle and multiple innervation might provide a clue as to whether there is a relationship between monoinnervating neuron and nervous trophic effect.

Dependence of intrauterine myogenic cell growth on nerve is not clear. In many cases, myogenic cells grow in the absence of innervation. In lumbrical muscle of the hind leg of rat embryos, clusters of undifferentiated presumed myoblasts are formed. Axons with local enlargement are found at these loci in close contact with these cells (Ross et al. 1987a). Nerve trunks are found near clusters of myoblasts in chicken hind limb (Butler and Cosmos 1981),

but whether the nerve terminals are in contact with these cells is not known. Mononucleated cells present on the surface of mouse myotubes also contact the terminals of nerve, which also innervates the myotubes (Duxson et al. 1986). Following injection of β-bungarotoxin to rat embryo, the number of mononucleated cells decreases (Ross et al. 1987b). In chicken embryo, muscle mass is remarkably reduced following aneural operation at the neural tube. But since muscle mass enlargement occurs in parallel with body growth, denervation does not necessarily result in muscle atrophy (Butler et al. 1986). Removal of neural tube, however, results in a decrease in the number of myotubes formed (Phillips and Bennett 1984).

The effect of nerve on embryonic muscle cell development and growth cannot be excluded, but the evidence is not conclusive.

2.2.4 Transmission of Nervous Trophic Stimulus

Following denervation, nerve stimulus is lost, and muscle fibers do not move. Gutman compared the effect of denervation and mechanical immobilization of innervated muscles (1962, 1964). In both cases, muscle atrophy ensues from an operation. Denervation induced the following: (a) increased sensitivity to ACh and expansion of the ACh-sensitive area around neuromuscular synapse (Kuffler 1943), (b) spontaneous fibrillation (Tower 1937a), (c) electrical excitability change (Erb 1868), (d) increase in glycogen deposition, and (e) increase in DNA content. Following immobilization, however, these effects were not seen. Based on these observations, Gutmann (1976) claimed that motor neuron has a trophic "effect" other than the effect to induce contraction of muscle fiber. Support for this claim finds forerunners of his research, for example, in Tower (1937a, b, 1939) and Eccles (1941). Gutmann did not use the terms trophic "factor" of "substance." However, there is a synaptic gap between motor nerve terminals and muscle fibers. Thus, the next question is what conveys the trophic stimulus for growth and maintenance from nerve terminals to muscle cells: does ACh serve as the trophic factor, or is there another specific trophic factor(s)?

Some researchers believe ACh to be the trophic substance. Denervation is possible not only surgically but also pharmacologically. Chronic administration of various inhibitors which prevent ACh from interaction with ACh receptor (AChR) results in muscle fiber atrophy: these are botulinum toxin and β-bungalotoxin which inhibit the release of ACh from nerve terminals, curare which inhibits ACh from binding to AChR, and hemicholinium-3 which inhibits uptake of choline by the nerve terminal, a substrate for ACh resynthesis (Thesleff 1960; Johns and Thesleff 1961; Hofmann and Thesleff 1972). Drachman (1974) showed that the effects of chronic botulinum toxin administration and surgical denervation are qualitatively identical in the following aspects: (a) histochemistry of muscle (Drachman and Romanul 1970), (b)

slowing of the twitch contraction of fast muscle (Drachman and Houk 1969), (c) sprouting of nerve fibers, (d) innervation of foreign nerves, and (e) choline extramuscular junction. These results may support the view that ACh acts as a trophic factor and suggest the possibility that the mechanical work prevents muscle from atrophying.

Experiments were performed to examine whether electrical stimulation of denervated muscle prevents atrophy, since the ACh hypothesis implies that excitation of cell membrane is essential for its maintenance; results showed electrical stimulation to be fairly effective (Thomson 1952). Direct stimulation of denervated muscles prevents AChR from expanding (Jones and Vrbova 1974) and inhibits the appearance of fibrillation (Lømo and Rosenthal 1972; Purves and Sakmann 1974) or innervation by other nerves (Jansen et al. 1973). However, these results are still considered insufficient for researchers to exclude the presence of a specific trophic factor released from the motor nerve.

The current view seems to favor the idea that nerve terminals release a specific trophic factor(s) other than ACh (Fernandez and Donoso 1988), despite the lack of conclusive evidence. This factor is usually thought to be synthesized in soma (nerve cell body), transported on the axonal flow to the nerve terminal, and released to the synaptic gap, to exert a trophic effect of muscle fibers. In this context, Davis' group found that denervation atrophy was prevented to some extent when sciatic nerve extract was injected at the locus of denervation or intraperitoneally (Davis and Kiernan 1980, 1981; Davis and Heinicke 1984; Davis 1985; Heck and Davis 1988). This extract was not replaced by Tf. Partially purified factor had a molecular weight of about 100 kDa and pI of 7.0 and was glycoprotein (Davis et al. 1985). Although still preliminary, these findings are considered by some investigators to suggest the presence of a neurogenic muscle trophic factor (Davis 1988).

In the embryonic stage of duck, destruction of the central nervous system results in reduced muscle development, while AChR blockade by α-bungalotoxin does not, suggesting the participation of a neurogenic trophic factor other than ACh (Sohal and Holt 1980).

Whether such a trophic factor is synthesized at nerve terminals like ACh or at soma poses a question. The biological significance of these two mechanisms is greatly different, since a single motor neuron should furnish the trophic factor to a large number of muscle fibers (see Sect. 5.1).

3 General Biology of Transferrin and Transferrin Receptor

3.1 Tf and TfR Molecules

Tf is a glycoprotein which binds two atoms of Fe per molecule and is present in serum and in tissue fluid such as cerebrospinal fluid and seminal fluid.

Certain molecular species are closely related to serum Tf (sTf) in molecular structure; these are ovotransferrin (ovoTf), present in avian egg white, and lactoferrin, present in milk (for review see Bezkorvainy and Zschocke 1974; Zschocke and Bezkorvainy 1974). Tf transports Fe to cells after binding to TfR which is located on cell membrane (Jandl et al. 1959; Jandl and Katz 1963). Since Fe is an important component of hemoglobin and an indispensable substance for cell proliferation, a large number of studies have been made using erythroid cells, cell lines, and cancer cells (for reviews see Aisen 1980; Trowbridge et al. 1984; Crichton and Charloteaux-Wauters 1987).

3.1.1 Tf Molecules – Their Synthesis and Presence

Tf is usually purified from serum and egg white. Fe-free apoTf is colorless, but when it binds with Fe, holoTf shows a characteristic salmon-pink color, the maximum light absorption wavelength of which is 465 nm. Tf is soluble in water, and its molecular weight is about 80 kDa including the sugar chains (for review see Bezkorovainy 1980).

Primary molecular structures of variousTf families have been determined in human sTf (MacGillivray et al. 1982, 1983), Lf (Mazurier et al. 1983; Metz-Boutigue et al. 1984) and chicken ovoTf (Williams et al. 1982a) by amino acid sequence analysis, and in human sTf (Uzan et al. 1984; Yang et al. 1984) and chicken ovoTf (Cochet et al. 1979; Jeltsch and Chambon 1982) by cDNA sequence analysis. Human Tf gene maps to chromosome 3q231–25 (Yang et al. 1984; Huerre et al. 1984). In chicken, one Tf gene is present per haploid genome and is composed of 17 exons (Cochet et al. 1979).

Tf is split into two similar parts by reduction alkylation or performic acid treatment (Jeppsson 1967). There is 41.7% amino acid sequence homology between these two domains (MacGillivray et al. 1982). String models of chicken ovoTf and human Lf have been reported in which many intramolecular disulfide bonds are present, and one Fe atom binds to each domain to form holoTf (Williams et al. 1982b; Baker et al. 1987).

Sugar chains of Tf from various mammalian and avian species are sialylated bianntenary glycans of the *N*-acetylgalactosamine type. Sugar chains of Lf are fucosylated while those of chicken and turkey ovoTf are neither sialylated not fucosylated (Spik et al 1985). Difference in the number of sialic acid residues is reflected in the difference in isoelectric points of Tf. Neuraminidase treatment of chicken sTf increases its pI, which finally coincides with the pI of asialoTf or ovoTf (Kimura et al. 1982). Despite the difference in pI, the biological nature of these forms of Tf is the same (Ii et al. 1982).

Tf binds various metals other than Fe; these are Cr, Cu, Mn, Co, Cd, Zn, Ni (Tan and Woodworth 1969), Sc (Ford-Hutchinson and Perkins 1971), V

(Cannon and Chasteen 1975), Ga (Woodworth et al. 1970), Pt (Stjernholm et al. 1978), Al (Martin et al. 1987), and La (Luk 1971). Some of these metals, albeit to a very minute amount, may contaminate conventional Fe compounds. Therefore, it is possible that certain effects of Tf which are usually ascribed to the role of Fe transported to the cells may sometimes be modified by trace amounts of these metals.

In adult animals, almost all Tf is synthesized in liver (for review see Bezkorovainy 1980). Tf mRNA and Tf synthesis are regulated by nutritional uptake of Fe (McKnight et al. 1980a). Tf is also synthesized in choroid plexus (Dickson et al. 1985; Bloch et al. 1987), estrogen-stimulated chicken oviduct, brain, pancreas, kidney (McKnight and Palmiter 1979; McKnight et al. 1980b), and testis (Skinner and Griswald 1980). In cultured tissue from mouse embryo, Tf is synthesized mostly in visceral yolk sac and liver and slightly in spinal cord, brain, ribs, lung, muscle, and heart (Adamson 1982; Meek and Adamson 1985). It is noteworthy that there are controversial reports on the synthesis of Tf in cultured neurons (Stamatos et al. 1983; Markelonis et al. 1985; see Sects. 8, 9).

Tf concentration in human adult plasma is about 2.5 mg/ml (Weippl et al. 1973; Bothwell et al. 1979), and about 40% – 50% of sTf is Fe saturated. In chicken sTf, Fe saturation is nearly 90%.

The percentage of Tfs with various pI values contained in chicken serum is dependent on developmental stage. The most acidic species of Tf, which is initially the least component, increases to become the most abundant species (Kimura 1983). Thus, the sialization of sTf may increase with time, since no isoforms with different amino acid sequences have been reported.

3.1.2 TfR Molecules and Anti-TfR Antibodies

TfR has been purified from cell membrane as a protein which can bind Tf or anti-TfR monoclonal antibody. TfR is a glycoprotein with molecular mass of 180 – 190 kDa including the sugar chain. Its isoelectric point ranges from 5.2 to 6.6, and it is composed of two homodimers of 94 kDa linked by a disulfide bond (van Bockxmeer et al. 1978; Witt and Woodworth 1978; Seligman et al. 1979; Hamilton et al. 1979; Wada et al. 1979; Enns and Sussman 1981). Molecular mass of chicken nerve TfR was reported to be 56 kDa; the reason for this large disparity is not known (Markelonis et al. 1985).

N and C terminals of TfR are located inside and outside of the cell membrane, respectively. The human TfR subunit is composed of 760 amino acid residues, and its molecular mass is calculated as 84.910 kDa. Of the 28 amino acids between the cysteines located at the 62nd and 89th amino acids, 14 amino acids are hydrophobic and other 14 unchanged, and this is considered to be a membrane-spanning peptide. Thus, the TfR monomer is composed of

three peptide parts of 61, 28, and 671 which are located inside the cytoplasm, in the cell membrane, and outside the cell, respectively. Cysteine at the 98th amino acid makes a disulfide link between the two subunits to form TfR protein. There is an amino acid sequence Lys-Arg-Lys starting from the 128th amino acid which must be attacked by trypsin to give a fragment with 70 kDa. Actually, treatment of cells with trypsin creates a TfR peptide fragment of 70 kDa. There is a binding site of monoclonal anti-human TfR antibody OKT9 on this fragment. It is considered that sugar antennas bind with Asp which is present as Asp-X-Ser/Thr. There are three putative sequences on the fragment present outside the cell (Schneider et al. 1982, 1983, 1984; Kühn et al. 1984; McClelland et al. 1984). Although TfR sequences deduced from cDNA lack a signal peptide (McClelland et al. 1984), it is suggested that the transmembrane segment serves in this capacity (Zerial et al. 1986).

Human genomic cDNA of TfR has been obtained (Kühn et al. 1984), as well as the gene maps to chromosome 3q262–q ter (Goodfellow et al. 1982; Enns et al. 1982; Miller et al. 1983; Plowman et al. 1983; Seligman et al. 1986). The relationship between Tf and TfR genes, both of which map to 3q, is not known. Monoclonal antibodies against human, rat, and mouse TfR (Bramwell and Harris 1978a, b; Aisenberg and Wilkes 1980; Omary et al. 1980; Haynes et al. 1981; Trowbridge and Domingo 1981; Trowbridge and Lopez 1982; Trowbridge et al. 1982; Jefferies et al. 1985) have been used for the studies described below.

3.2 Expression of TfR

TfR is present not only on cell membrane but also inside the cell. Its expression is related to the cellular Fe requirement of the cells. The rate of TfR synthesis is dependent on the free Fe pool in the cells (for review: Huebers and Finch 1987; Ward 1987). In other words, the more Fe that is required by cells, the more TfR is expressed.

3.2.1 Cell Growth and Fe

Requirement of Fe for cell proliferation has been known for a rather long time. In 1961, Neuman and Tytell found that Tf is one of the most important constituents of serum-free medium for rat carcinosarcoma cell line. Tf as a growth promoter has since been repeatedly discovered in cultures of mouse spleen cells (Vogt et al. 1969), lymphocytes (Tormey and Mueller 1972; Phillips and Azari 1974), and rat GH3 cells (Hayashi and Sato 1976). The importance of Tf was widely recognized in the reviews of Barnes and Sato (1980a, b). The least effective Tf concentrations are 0.1 and 3 μg/ml, and

those producing maximum effects are 10 and 30 μg/ml in rat and chicken myogenic cell growth, respectively (Shimo-Oka et al. 1986).

One of the most important roles of Tf is to donate Fe to red cells as a material for hemoglobin (for review: Aisen 1980). The role of Tf in cell proliferation is also to donate Fe. It is clear that what is essential in FeTf is Fe, since FeTf can be replaced by Fe ion, hemin (Wu and Sato 1978; Hasegawa et al. 1981; Verger et al. 1983), and even larger molecules such as hemoglobin and myoglobin which do not have specific receptors on the cell surface (K. Saito, personal communication). This is also supported by the fact that Tf binding metals other than Fe (Saito et al. 1982) and heme-related substances which do not contain Fe such as bilirubin do not replace FeTf. It must be emphasized that Tf serves as an Fe donor; Fe can replace FeTf while apoTf has no biological effect. There has been no report that Tf protein plays a role other than in Fe transport, except its involvement in the reduction of Fe by the transmembrane electron transport system (Sun et al. 1987a, b; Low et al. 1987). Therefore, the transducing effects of a ligand which are usually considered in various growth factors are not considered in the case of Tf.

Addition of the Fe chelators, parabactin, compound II, or desferoxamin to Fe-free culture medium gives rise to a reversible cessation of the cell cycle and accumulation of cells at the G1-S boundary (Lederman et al. 1984; Cavanaugh et al. 1985; C. Yoshida-Noro, personal communication).

The mechanism by which Fe is involved in the cell cycle is fairly well understood, although some crucial experiments are lacking. In the absence of Fe, provision of material for DNA synthesis is blocked. Ribonucleotide reductase which catalyzes deoxidization of NDP (N: A, G, or C) to form deoxy-NDP requires Fe for its activity (Reichard 1978; Thelander and Reichard 1979). dNDP is phosphorylated by a kinase to form dNTP, which is then used for DNA synthesis. Ribonucleotide reductase is composed of two kinds of subunits, M1 and M2, forming a tetramer. One M2 subunit binds one atom of Fe (Thelander et al. 1985). The amount of M2 subunit increases in S phase, and decreases to an almost undetectable level in resting cells (Engstrom et al. 1985; Neckers and Nordan 1988). These findings are compatible with the fact that cells are accumulated at the G1-S boundary in Fe deficiency. Further, cellular ribonucleotide reductase activity decreases in the absence of Tf in culture medium and increases rapidly on provision of Tf (C. Yoshida-Noro, personal communication).

The number of TfR on cell membrane changes depending on the phase of cell cycle, being high in S and G2 phases but low in G1 phase (Rovera et al. 1982). In M phase, the number decreases remarkably, and endocytosis and exocytosis of the Tf-TfR complex are seldom observed (Warren et al. 1984; Sager et al. 1984).

3.2.2 Regulation of TfR Synthesis and Distribution

The number of TfR is large in rapidly proliferating cells such as cancer cells and some cell lines, and undetectable in fully differentiated erythrocytes in peripheral blood (Jandl and Katz 1963; Hamilton et al. 1979; Enns et al. 1981). It is possible to differentiate various leukemia cell lines by treatment with some drugs (Yeh et al. 1982; Besancon et al. 1985). When M1 cells, for example, are induced to differentiate by dexamethasone treatment, their number decreases from 4.5×10^5 to 2.85×10^5 (Tei et al. 1982).

The number of TfR increases when cell proliferation is promoted on fresh medium, by treatment of T cells with interleukin 2 or on liver regeneration (Sawyer and Krantz 1986; Testa et al. 1986; Hirose-Kumagai et al. 1984). On the other hand, developing erythroid cells which no longer proliferate retain a large number of TfR (Horton 1983; Iacopetta et al. 1982); this is because these cells synthesize hemoglobin and require Fe. After cessation of hemoglobin synthesis, many vesicles which contain TfR and Tf are expelled from the cells (Pan and Johnstone 1983). Another known example of the retention of numerous TfR after cessation of proliferation is polynucleated muscle cells. This is explained below.

It is anticipated that the number of TfR expressed on the cell surface changes depending on the requirement of Fe of the cells. When ferric ammonium citrate or hemin is added to the medium and taken up by the cells, cell surface TfR decreases to less than 50% within 24 h. This is reversed following Fe removal from the medium. When desferoxamin is added to the medium, the TfR number increases (Ward et al. 1982, 1984; Testa et al. 1982; Louache et al. 1983, 1984; Mattia et al. 1984; Klausner et al. 1984b; Rouault et al. 1985; Rudolph et al. 1985). It is possible that desferoxamin treatment results in a change in distribution of TfR inside the cell and on the cell surface (Bridges and Cudkowicz 1984). However, it is clear that de novo synthesis of TfR increases, as shown by the increase in TfR mRNA and cyclohexamide-induced inhibition (Mattia et al. 1984; Rao et al. 1985; 1986; Rudolph et al. 1985). Under these conditions, the half-life of TfR and the ratio of surface TfR to intracellular TfR does not change, and thus a change occurs in the number of total TfR (Rao et al. 1985).

Fe regulates directly the TfR gene. The promoter is located from 5′ flanking sequence to the first intron and there are several protein binding sites (Miskimins et al. 1986). The sites regulated by Fe are located at the 3′ noncoding region and the 5′ region (Owen and Kühn 1987; Casey et al. 1988a). At the 3′ noncoding region, there is an Fe-responsive element having five hairpin structures (Casey et al. 1988b).

3.3 Tf Transportation into Cells

Soon after the discovery of TfR, the mechanism by which Fe is donated to cells was studied, and various types of evidence was gathered suggesting the internalization of Tf together with TfR (Morgan 1964; Morgan and Baker 1969). It has been established that Tf first binds TfR, and that this complex is endocytosed and then returns to the cell surface leaving Fe inside the cell (Karin and Mintz 1981; for review: May and Cuatrecasas 1985, and cf: Morley and Bezkorovainy 1985). During this internalization cycle, the Tf molecule remains intact (Kimura et al. 1985). This is an exceptional case because the internalized ligand is usually digested inside the cell in trophic factors other than Tf (Brown et al. 1983). The function of Tf for cell growth is widely believed to be in its delivery of Fe to cells, and the transducer mechanism on the cell surface to which the effects of various other growth factors are ascribed is not considered to be valid in Tf.

3.3.1 Class Specificity in Tf-TfR Binding

It is known that Tf from specific animal species works on cells from specific species (Tormey and Mueller 1972; Verhoef et al. 1973; Sephton and Kraft 1978; Galbraith et al. 1980; Imbenotte and Verber 1980; Coll and Ingram 1981). Recent studies clarified that the specificity of Tf is class dependent. Mammalian Tf acts on mammalian cells but not on avian cells, and avian Tf acts on avian cells but not on mammalian cells (Ozawa and Hagiwara 1981; Hagiwara and Ozawa 1982; Ozawa 1985; Beach et al. 1985; Shimo-Oka et al. 1986). This peculiar specificity is called "class specificity," although there are a few exceptions. Bovine and dove Tf at high concentrations cross-react beyond their classes. This specificity is dependent on the affinity of Tf to TfR (Shimo-Oka et al. 1986; Tsavaler et al. 1986; Penhallow et al. 1986). Class specificity is also shown in Tf-containing serum (Hagiwara and Ozawa 1982).

Presence of this specificity makes study of Tf easy because the effect of specific Tf can be shown in a medium containing Tf which does not bind TfR of the cells used. For example, chicken myogenic cells do not grow in a medium composed of synthetic medium and horse serum, since horse Tf is impervious to chicken TfR. Further, serum contains apoTf which can be expected to sequester Fe ion contaminated in a culture medium.

3.3.2 Internalization and Recycling of Tf

Crichton and coworkers showed that when rat embryo fibroblasts were incubated with [^{59}Fe, ^{3}H] Tf, Fe was accumulated substantially inside the cells. Tf protein moiety, however, was little accumulated in the cells but, instead, in the medium. They also showed that drugs suppressing lysosomal function such

as chloroquine methylamine suppressed the Fe uptake (Octave et al. 1979, 1981, 1982).

Karin and Mintz (1981) provided the internalization cycle model described above (see Sect. 3.3). Their work was followed by various morphological studies (Harding et al. 1983; Hopkins and Trowbridge 1983; Pearse 1982; Enns et al. 1983; Iacopetta et al. 1983; Iacopetta and Morgan 1983b). Tf first binds to TfR which is scattered on the cell membrane. The Tf-TfR complexes are gathered together in clathrin-coated pits and then move into the cells packed in coated vesicles. These coated vesicles and intracellular organellae combine to form multivesicular bodies (MVB). MVB is about 100 nm or more in diameter and is visible under an optical microscope when stained immunohistochemically.

Fe is removed from Tf by the acidification in MVB. In vitro Tf binds Fe very tightly at neutral pH and release Fe at acidic pH. These characteristics are also shown with Tf bound to TfR on the cell surface on changing pH of the medium. The critical pH for association and dissociation of Fe seems to be between 5.5 and 6.0. ApoTf binds well to TfR at pH lower than 6.5 but does not bind at higher pH, while holoTf can bind to TfR at either pH (Dautry-Varsat et al. 1983; Morgan 1983; van Renswoude et al. 1982; Klausner et al. 1983a, b). pH in MVB is assumed to range from 5 to 5.8 (van Renswoude et al. 1982). Therefore, it is plausible that Fe is released in MVB leaving the apoTf-TfR complex. When an alkalizing agent such as chloroquine, methylamine, NH_4Cl, or monensin is donated to cells, Fe release inside the cells is inhibited, but Tf cycling is not disturbed (Rao et al. 1983). The nature of acidifying organellae is not known. Although they were once considered to be lysosomes, van Renswoude et al. (1982) reported that this is not the case, showing dissociation of MVB from lysosome marker enzyme β-hexosaminidase by Percoll gradient fractionation.

No definitely conclusive evidence has been derived as to how Fe dissociated from Tf is transferred to the molecules with which Fe finally binds.

MVB appears to be associated with the Golgi apparatus (Willingham et al. 1984) and associated MVB resembles a "cap" (Iacopetta et al. 1983). Eventually, MVB returns to the cell surface and it opens to the medium present outside the cells where pH is more than 7.0. Thus, the apoTf-TfR complex is exposed to the medium. ApoTf, the affinity of which to TfR is very low at neutral pH, leaves the cell surface (Harding and Stahl 1983; Hanover et al. 1984).

In many ligands other than Tf, internalized ligands do not return but are catalyzed at lysosomes. Tf once internalized and recovered remains intact in molecular weight (Karin and Mintz 1981; Ciechanover et al. 1983a), isoelectric point, the number of sialic acids bound to the terminals of sugar chain, Fe binding, and biological activity (Kimura et al. 1985). Thus, during the cycling, Tf is not changed except to lose Fe.

Time required for one cycle differs depending on the reports (Klausner et al. 1983a, b; Hopkins and Trowbridge 1983; Mulford and Lodish 1988), but the mean cycling time is roughly 16 min. The amount of Tf and Fe taken up by mononucleated cells is about $1-3\times10^4$ molecules/cell per minute and $2-6\times10^4$ atoms/cell per minute, respectively (Ciechanover et al. 1983b; Iacopetta and Morgan 1983a).

TfR is distributed in both cell surface and intracellular space, and the distribution ratio of TfR in the two locations is relatively 1:2 (Ciechanover et al. 1983a) or 1:4 (Klausner et al. 1983b).

The question of whether internalization of TfR occurs without Tf binding has not been resolved. One possibility is that TfR moves "up" to the cell interior, and "down" to the cell surface irrespective of whether it binds Tf, as an escalator moves irrespective of whether or not it carries passengers (escalator hypothesis; Hanover et al. 1985; Watts 1985; Stein and Susman 1986). Another possibility is that the binding of Tf to TfR is a prerequisite for the TfR movement; in other words, TfR moves like an elevator which works only when there are passengers in it (elevator hypothesis; Enns et al. 1983; Klausner et al. 1984a). In any case, the cycle itinerary depends on energy supply since it does not occur at low temperature or in the presence of NaCN or 2-deoxyglucose, a nonmetabolizable equivalent of glucose (Morgan and Appleton 1969; Ciechanover et al. 1983a).

It is plausible that cytoskeleton plays an important role in the cycling itinerary since colchicin, vinblastin, strychnine, and heavy water which inhibit the formation of cytoskeleton also inhibit Tf and Fe uptake (Hemmaplardh et al. 1974).

Another factor which may be related to the TfR cycling is protein kinase C (for review: Nishizuka 1984, 1986). The potent carcinogenic substance 12-*o*-tetradecanoyl-phorbol-13-acetate (PTA) activates the kinase (Castagna et al. 1982). When PTA, phorbol dibutylate, or 4β-phorbol 12-myristate 13-acetate (PMA) is applied, the number of cell membrane TfR decreases within ten min ~ several hours and returns to the original level with another several hours (Rovera et al. 1982; May et al. 1984, 1985; Klausner et al. 1984a). Since this phenomenon cannot be blocked by cycloheximide, an inhibitor of protein synthesis, this change may be due to an alteration in the distribution of TfR (May et al. 1984). The presence of PMA and phorbol dibutylate occasions the promotion of Tf and Fe uptake, and the "escalator" phenomenon is also observed (Klausner et al. 1984a; Hebbert and Morgan 1985). α-Phorbol, which is not a carcinogen, does not promote this cycle.

TfR has a phosphorylated site by kinase C at serine 24 which is present at the intracellular domain (Davis et al. 1986; May et al. 1986). However, in transgenic cells in which the serine residue is replaced by unphosphorylated alanine, threonine, or glycine residue, TfR internalization is promoted by phorbol ester (Zerial et al. 1987; Davis and Meisner 1987; Rothenberger et al.

1987; McGraw et al. 1988). Therefore, phosphorylation of TfR may not be essential for the internalization.

4 "Transferrinology" in Muscle Cell Growth and Maintenance

Effects of Tf on myogenic cultured cells have been investigated mainly by two groups who have different working hypotheses. The first group sought to obtain effective substances from embryo extract (EE), a simple extract of chicken embryo that promotes myogenic cell growth (for review see Ozawa et al. 1983). The second group had the very ambitious purpose of obtaining a muscle trophic factor(s) released from the nerve terminal (see Sect. 2; for review see Oh and Markelonis 1984).

4.1 From Embryo Extract to Tf

The history of tissue (cell) culture in the early twentieth century was one of determining good culture media in which cells could grow well. Alexis Carrel (1913), who laid the foundation of the present-day culture method, found that embryonic juice (almost equivalent to EE) had a potent growth-promoting action for fibroblasts. As a surgeon he tried to purify this active substance to use as a healing remedy for injuries. His attempts were not successful, however, because general biochemistry itself was still in its infancy at that time. Similar trials were made later (Kutsky and Harris 1957; Kutsky 1959; Coon 1966). In 1972, de la Haba and Amundsen fractionated EE into two fractions with a molecular weight cut corn, which allows molecules smaller than 50 kDa filtrate through the membrane on centrifugation leaving molecules larger than 50 kDa in the unfiltrable fractions, but purification of the active principles had to await further research.

Chicken myogenic cells grow well in a medium composed of synthetic medium, horse serum, and a small amount of chicken EE (Konigsberg 1963). When EE was removed from the medium, chicken cells did not grow well. When simple extracts of various embryonic organs were added in place of EE, each extract showed a stronger or weaker growth-promoting effect. Chicken embryonic and adult sera were the most effective of these (Ozawa and Kohama 1973, 1978a, b; Kohama and Ozawa 1973, 1978). Ozawa and his coworkers used serum as the starting material and obtained a muscle trophic substance; they later obtained the same from EE (Kimura et al. 1981, 1982; Ii et al. 1982). Other groups also tried to purify muscle trophic substances and obtained crude fractions (Slater 1976; Jabaily and Singer 1978).

The muscle trophic substance prepared from serum or EE was identified as Tf on the basis of the following evidence. When the substance was com-

pared with authentic Tf prepared from serum and egg white, they had the same molecular weight, color and absorption spectrum, Fe binding capacity, isoelectric point, immunological properties, and specific activity to promote myogenic cell growth (Kimura et al. 1981, 1982; Ii et al. 1981, 1982). Tf from chicken serum and EE can be separated by electrofocusing into three isoforms due to the difference in their respective numbers of neuraminic acids attached to the termini of sugar chains. In spite of the difference, these three isoforms showed the same trophic activity (Kimura et al. 1982; Ii et al. 1982).

Tf was the first trophic factor purified from EE. Thereafter, hypoxanthine (Ii et al. 1985) and fibroblast growth factor-like factor or heparin-binding growth factor (Ii and Ozawa 1985; Kimura et al. 1989) were also derived from EE. There are very probably many other yet undiscovered factors in EE which promote cell growth.

Convenient and reliable bioassay systems are a prerequisite for the study of a physiologically active substance. Muscle cells grow not only by proliferation but also by protein accumulation (see Sect. 2), and both factors can be used as markers for the bioassay. Although increase in cell number is commonly used as a marker for growth of various kinds of cells, in myogenic cells cell fusion makes this impossible. Further, contamination of fibroblasts is almost inevitable in primary myogenic cell culture. Fibroblasts usually start to proliferate almost coincidentally with the time of myogenic cell fusion (K. Kohama, unpublished data). Therefore, the total amount of DNA is not a suitable marker for myogenic cell growth. Although there have been some cases of the selective counting of the number of nuclei contained in myogenic cells (Ozawa and Kohama 1973, 1978a; Kohama and Ozawa 1977; Doering and Fischman 1977), this method is little used today because it is rather troublesome, and because it is not easy to discriminate myoblasts from fibroblasts on the basis of their cell shape in mass culture (Buckley and Konigsberg 1974; Holtzer et al. 1975; Konigsberg et al. 1978).

Determination of protein accumulation is another marker for bioassay. Usually, a muscle-specific protein is measured, such as myosin (Paterson and Strohman 1972). Creatine kinase activity has also been used (Shainberg et al. 1970; Turner et al. 1976; Doering and Fischman 1977; Ozawa 1978). The creatine kinase activity of a primary muscle cell culture has been shown to be almost exclusively derived from myotubes by separate assay of the activities in myotubes and mononucleated cells (myoblasts and fibroblasts) (Ohtsuki and Ozawa 1977). Creatine kinase activity is linearly correlated with the amount of total protein accumulated (Hagiwara et al. 1989a). Formation of large myotubes with high creatine kinase activity requires the participation of many myoblasts. This means that creatine kinase activity reflects the combined results of cell proliferation before fusion and the accumulation of proteins following fusion.

4.2 Effects of Tf on Muscle Cells

Not all the characteristics of TfR and its relation to Tf described in Sect. 3 have been examined in myogenic cells. However, the evidence available suggests that most of those characteristics are shared by muscle cell TfR.

Myoblasts, myotubes, and muscle fibers have TfR on the cell surface (Fava et al. 1981; Hasegawa and Ozawa 1982; Jefferies et al. 1985; Shimo-Oka et al. 1986; Stamatos and Fine 1986; Sorokin et al. 1987; Oh et al. 1988). The muscle Tf-TfR system has class specificity (Ozawa and Hagiwara 1981). Kinetics of the cycling itinerary in myogenic cells is almost the same as that described for other cells in Sect. 3.2 (Stamatos and Fine 1986; Y. Hagiwara, unpublished data). Phorbol ester PMA increases the rate of Fe uptake by myogenic cells (Sorokin et al. 1988), and that Fe is the essential moiety also true of myogenic cells. Fe-containing substances such as Fe citrate, hemin, myoglobin, and hemoglobin (at higher concentrations) replace Tf in promoting myogenic cell growth (Hasegawa et al. 1981; Saito et al. 1982; Kayoko Saito, personal communication). Some transition metals which are able to bind to Tf (Tan and Woodworth 1969) are ineffective for cell growth in either free ion or Tf-bound form (Saito et al. 1982).

One of the characteristics of myoblasts is that proliferation is promoted by Tf; for example, chicken primary myoblast and cell line L6 proliferate in a dose-dependent manner with regard to Tf (Ozawa and Kohama 1978a; Shimo-Oka et al. 1986). In the absence of Tf, L6 cells accumulate at the G1-S border (C. Yoshida-Noro, personal communication). These characters are compatible with those of other kinds of cells.

Myotubes grow by accumulating muscle-specific proteins which serve as hallmarks of muscle differentiation. Further, isoform changes of these various proteins, cross-striation formation, and spontaneous twitching take place during myotube growth in vitro (Turner et al. 1974; Perriard et al. 1978; Toyota and Shimada 1983). Tf or Fe contributes to these molecular, morphological, and physiological differentiations. The expression of elements necessary for muscle contraction and energy metabolism, such as AChR (Markelonis et al. 1982b), myosin (Matsuda et al. 1984a), Ca-stimulated actomyosin ATPase, myofibrils, membrane excitability, contractility, phosphorylase kinase, and changes in creatine kinase isoforms (Hagiwara et al. 1987) and in collagen isoforms (Gerstenfeld et al. 1984) are all dependent on the presence of Tf. Dibucaine, a local anesthetic and protein kinase C inhibitor (Mori et al. 1980), suppresses myogenic cell growth and differentiation (Hagiwara et al. 1985) and Fe uptake through retardation of Tf-TfR cycling (Y. Hagiwara, unpublished data).

For the maintenance of well-developed myotubes, Tf is absolutely essential (Ozawa 1977; Markelonis and Oh 1979; Ozawa and Hagiwara 1982). When large myotubes are cultured without Tf, morphological growth and the accu-

mulation of protein such as creatine kinase are arrested. These myotubes then deteriorate to show many fatty droplets, vacuoles, and atrophy accompanied by loss of cross-striations. These changes are essentially degeneration. Finally, they become lagged and die away. In other words, myotubes degenerate under Tf-deficient conditions. Myoblasts and undeveloped myotubes are relatively resistant to Tf deficiency. When atrophied myotubes and remaining myoblasts and undeveloped myotubes are refed with Tf, large myotubes are formed which eventually show cross-striations and spontaneous twitching. These may be myotubes recovered from atrophy and those newly formed by the fusion of myoblasts (Ozawa 1977; Ozawa and Hagiwara 1982).

Tf deficiency results in a decrease in protein synthesis but has little influence on protein degradation (Markelonis et al. 1980; Shoji and Ozawa 1986). Synthesis of total RNA and mRNA of myosin heavy and light chains is reduced, but their degradation rate does not alter. Less RNA is synthesized in run-off transcription in nuclei from Tf-starved myotubes than from Tf-supplied myotubes (Shoji and Ozawa 1985a, 1986).

How Fe transported by Tf effects the transcription has not been completely elucidated. Fe at 100 μM stimulated RNA polymerase activity (Shoji and Ozawa 1985b). However, such a high concentration of ferric ion would not be present under intracellular circumstances. On the other hand, the transcription factor AIII for the initiation of transcriptions has recently been proved to require Zn, which cannot be replaced by Fe (Klug and Rhodes 1987). Since ZnTf is also transported into lymphocytes (Phillips and Azari 1974), and conventional Fe salt reagents usually contain a minute amount of Zn, the possibility cannot be excluded that the effects of this trace metal may modify the effects of Tf which are ascribed to Fe.

The requirement of Fe for myotubes and muscle fibers in vivo must be emphasized in relation to their internal respiration. O_2 transported by hemoglobin is donated to myoglobin to be stored in muscle cells. In man, 11.4% and 8.4% of functional and total Fe, respectively, are present as myoglobin (Bothwell et al. 1979). Myoglobin content is greater in red muscle cells than in white muscle cells. Synthesis of myoglobin starts early in muscle development (Low and Rich 1973; Kagen et al. 1974; Heywood et al. 1973, 1974). In rat, red muscle cells differentiate about 2 weeks after birth under the influence of motor neurons (Rubinstein and Kelly 1981), and a large amount of Fe must be transported into the muscle cells during this differentiation. Fe transported on the vehicle of Tf may be the source of myoglobin-bound Fe (Sorokin and Morgan 1988). Other respiratory elements in mitochondria also contain Fe, albeit in small amounts (Keilin 1966).

The kinetics of Fe in the metabolism of these Fe-binding proteins is not known, nor is it known whether Fe is reused in individual cells although it is believed that individual animals do reserve most Fe for reutilization. Judging from the effects of Tf removal on myotubes in vitro, these cells may easily

release Fe or may put Fe into unutilizable compartments and thus require a continuous supply for their maintenance (see above). Mature muscle fibers also express TfR on the cell surface and intracellularly (Jefferies et al. 1985; Y. Gotoh et al., unpublished data). Further, TfR is strongly stained immunohistochemically in muscle fibers which are also stained with succinic dehydrogenase, a mitochondrial marker (Gotoh et al., unpublished data). This suggests that mature muscle fibers require an Fe supply to maintain normal activity, and this is compatible with the evidence that more TfR is expressed when more Fe is required (see Sect. 3.3).

4.3 From Nerve Extract to Sciatin

The hypothesis that a muscle trophic factor is released from the motor nerve terminal has attracted many researchers (for review see Oh et al. 1988). Oh and his coworkers reported the presence of factors in extract of spinal cord and peripheral nerves which promote myogenic cell culture in the presence of EE. Expression of AChE activity is strongly enhanced in vitro by innervation, coculture of spinal cord tissue, and addition of brain and spinal cord extract to culture medium (Oh and Johnson 1972). Thereafter, Oh's group reported several factors extracted from brain and spinal cord (Oh 1975, 1976; Oh and Markelonis 1978; Markelonis and Oh 1978). The molecular weights of these substances differed greatly among their reports, ranging from about 20 kDa to more than 300 kDa. Although they later claimed that all of these substances were Tf (Markelonis and Oh 1987), it is difficult to accept this view. Further, since their effects were observed in the presence of extract from embryos free from the brain and spinal cord which would have contained a large amount of Tf, the effect may be ascribed to substances other than Tf. For these reasons, their work prior to 1979 is not considered here.

Oh and his coworkers (Markelonis and Oh 1979) isolated a substance from chicken sciatic nerve that they termed sciatin. They conducted their work under the hypothesis (here referred to as the sciatin concept) that sciatin is a neurogenic muscle trophic factor released from motor nerve (see Sect.1). This substance has a molecular weight of 84.6 kDa and promotes myogenic cell growth when added to culture medium (Markelonis and Oh 1979; Markelonis et al. 1980). Chicken sciatin binds with concanavalin A specifically, suggesting that it is a glycoprotein; it is a mixture of four kinds of protein with different isoelectric points. Sugar chains which account for about 11% of the molecular weight contain no detectable *N*-acetylneuraminic acid. Sciatin shares 2.6% of the soluble protein of sciatic nerve. Neurons in the cerebral cortical region and spinal cord and sciatic nerve are immunohistochemically stained by anti-sciatin antibody. Various other tissues including muscle and liver, the latter being the principal site of Tf synthesis, are not stained (Oh et al. 1981).

Sciatin promotes protein synthesis but does not affect protein degradation in cultured myogenic cells. It promotes expression of AChR and ACh esterase (AChE) on the cell surface (Markelonis et all 1982b; Markelonis and Oh 1981). The presence of sciatin in EE is confirmed by the fact that anti-sciatin antibody treatment eliminates the trophic activity of EE (Oh and Markelonis 1980).

Sciatin content is sciatic nerve is about ten times that in the cerebral hemisphere on a protein basis. When sciatic nerve is ligated with silk thread, about six-fold more sciatin is accumulated upstream of the nerve control nerve. When adult chicken posterior latissimus dorsi muscles with their nerves were dissected out and placed in oxygenated Ringer's solution and the nerve stimulated, the solution contained 1.35 times more sciatin than unstimulated control (Oh and Markelonis 1982b). These work was done using anti-sciatin antibody. Most of their work on sciatin is summarized in the 1984 review of Oh and Markelonis.

One confusing piece of evidence for the sciatin concept is that extracts from normal and degenerated sciatic nerve are similarly effective in morphological maturation and long-term maintenance of myogenic cells in vitro (Oh et al. 1980).

Shortly after the appearance of Oh's report, Popiella, who once worked on the denervation problem using *Ambystoma* embryos (Popiela 1976, 1977), also obtained a substance with a molecular weight of 80 kDa from chicken ischiadic-peroneal nerves of the thigh region, the muscle trophic activity of which decreased at lower pH (Popiella 1978; Popiella and Ellis 1981; Popiella et al. 1982). These findings seem to confirm the sciatin concept.

Researchers in related fields, especially those concerned with denervation problem, have shown great interest in sciatin, hoping that the neurogenic muscle trophic substance which has long been sought has at last been found.

4.4 Sciatin is Tf

Sciatin was, however, shown to be very close to Tf. Their respective molecular characteristics and biological effects are very similar (Ii et al. 1981), and the muscle trophic effect of sciatic nerve extract is completely lost when treated with anti-sTf antibody (Ozawa 1981). Then, Oh, Markelonis, and their coworkers published papers stating that sciatin is a Tf-like protein. It has an amino acid composition strikingly similar to that of ovoTf, and the terminal 20 amino acid sequences of the two are identical. The two substances are physicochemically very close in the following aspects: molecular weight, sedimentation coefficient, diffusion constant, axial ratio, and isoelectric point. When sciatin binds with Fe, it becomes salmon pink in color (Markelonis et al. 1982a). Serum Tf is isolated by the same protocol used to purify sciatin.

Tf is indistinguishable from sciating in double immunodiffusion precipitin reaction of anti-sciatin antibody (Oh and Markelonis 1982a). Similar reports were made by Popiella and his coworkers (Beach et al. 1983; Popiela et al. 1984). Today, Oh's group appears to look upon sciatin as neurogenic Tf (Markelonis and Oh 1987; Oh et al. 1988).

Most of the important results presented above regarding sciatin were obtained using a variety of immunological methods. Unfortunately, the antisciatin antisera used were not characterized as excluding the nonspecific binding of the antibodies. One of the most important results is the immunohistochemical demonstration of axonal Tf. Although the antisera stained the axon, the antibody which had been pretreated with chicken plasma acetone powder also stained the axon (Oh et al. 1981). Since Hagiwara et al. (1981) showed that acetone powder of chicken serum contains a large quantity of native Tf, and K. Hayashi did not succeed in staining rat sciatic nerve axon with rat anti-sTf antibody (personal communication), it is likely that the antisciatin antisera contained antibodies against other components of the nerve. The antigen sciatin is prepared from peripheral nerve, so that even a small amount of contaminant with strong antigenicity may bring about antibodies that give these confusing results. This may also be true of other results obtained using these antisera.

5 How Tf Is Provided to Muscle Cells

Sciatin thus was shown to be Tf, and today Oh and his coworkers do not necessarily seem to view Tf as a neurogenic muscle trophic factor which works physiologically (Oh et al. 1988). However, the sciatin concept remains, and some reviewers today show keen interest in it (Mescher and Munaim 1988; Ochs 1988).

It is therefore important to consider whether a motor neuron serves as a donor of Tf or Fe to the muscle fibers that it governs. The data reported show that this is not the case; instead, Tf is provided to muscle from tissue fluid.

5.1 Does Tf Come from Motor Neurons?

In 1964, Eccles described the criteria which a neurotransmitter should fulfill in his famous monograph, *The Physiology of Synapses*:

> The substance must exist in sufficient quantities in the presynaptic terminals, which must also contain a synthesizing enzyme system; stimulation of the presynaptic nerves must release the substance in adequate quantities from the presynaptic terminals; the action of the substance on the postsynaptic cell must be identical with that of the synaptic action, particularly when applied by microelectrophoretic techniques; these should be an inactivating enzyme system in the region of the synaptic cleft.

These criteria apply for a substance which is released from a nerve terminal, such as a neurotransmitter. If Tf is a neurogenic trophic factor, it should fulfill similar criteria. Therefore, let us examine whether enough Tf can be provided from the nerve terminal to furnish the amount of Fe required by muscle fibers.

5.1.1 Production of Tf in Neurons

Experimental evidence on whether nerve cells in vitro can synthesize Tf is controversial. A neuron-rich culture from chicken synthesized Tf (Stamatos et al. 1983). However, Oh and his coworkers did not observe synthesis of Tf in chicken spinal cord cell culture (Markelonis et al. 1985). They state, on the other hand, that Tf is transported in the axonal flow (Oh et al. 1981; Oh and Markelonis 1982b), but they do not show the source from which Tf originates. The Tf gene is present on chromosome 3p in man (Yang et al. 1984; Huerre et al. 1984), and Tf mRNA is transcribed in the nucleus but not in mitochondria present at the nerve terminal. In nerve cells, polysomes are found solely in soma, and only occasionally are aberrant polysomes present in axon hillocks (Peters et al. 1970). If Tf is synthesized in neuron, the site for synthesis must be soma. It may be transported by axonal flow to the nerve terminal for storage and serve as a muscle trophic factor. It should be emphasized here that ACh is synthesized at the nerve terminal and stored in an amount sufficient to transport the nerve signal to a muscle fiber. Since each branch of the motor axon has a role, a single motor neuron can regulate a large number of motor fibers without the load being unbearable.

Let us calculate how much amount Tf would be required to provide Fe to muscle fibers by a motor neuron. The exact size of each motor neuron is not known. One example of a cross-sectioned area of a motor neuron is 8246 μm^2 (Kojima et al. 1972). If the motor neuron is spherical, the radius should be about 52 μm. Since its actual shape is very complicated (Poritsky 1969), let us set r (radius) at 100 μm, and thus the calculated surface area is about 126000 μm^2. This assumption is reasonable since the surface area of the largest motor neuron of cat is assumed to be 98000 μm^2 (Barrett and Crill 1974). The diameter of muscle fibers of man ranges from 61 to 69 μm in men and from 42 to 53 μm in women (Dubowitz et al. 1973) and is set at 40 μm for calculation. The length is put 10 cm, and the shape of the fiber is approximated by a cylinder. The number of muscle fibers which are controlled by one motor neuron (the neuron and muscle fibers together are called motor unit) ranges from 140 to 2000 (Burke 1981) and is set at 100 for calculation. Under these conditions, the ratios of volume and surface area of a group of muscle fibers in a motor unit to those in the motor neuron are about 1.2×10^4 and 2×10^4, respectively. These values may be underestimated. If a motor neuron provides Tf for the muscle, it should support muscle cytoplasm

in a volume 1.2×10^4 times greater than its own. For this purpose, a neuron would have to synthesize a large amount of Tf protein and collect Fe from outside of the cell.

Motor neurons are present in the spinal cord, which is covered with the pia mater and floated in cerebrospinal fluid present in a sheath of the dura mater. Nerve axons from the neurons contact tissue fluid after penetrating the dura mater, but almost all parts of the axons are covered by a myelin sheath of Schwann's cells. Cerebrospinal fluid contains about 10–60 μg/ml Tf (Davson et al. 1987) which is produced at the choroid plexus (Dickson et al. 1985; Bloch et al. 1985, 1987), and blood vessels penetrate the spinal cord. However, it is difficult for Tf to reach motor neurons because there are barriers between the cerebrospinal fluid and neurons and also between blood and neurons. Tf contained in these fluids may not be able freely to contact motor neurons (for review see Davson et al. 1987). Actually, neurons may obtain Fe from these fluids at least for maintenance of their internal respiratory system. However, if a neuron collects Tf through TfR on its surface to provide Fe for muscle fibers, which contain a large amount of myoglobin, the efficiency of TfR to collect Tf must be enormous.

The metabolic rate of Fe in muscle fiber is not known. In a man of 75 kg body weight, 320 mg Fe, i.e., about 8.4% of all Fe present in the body is in the form of myoglobin (Bothwell et al. 1979). Daily Fe requirement is about 0.5 mg, and thus about 0.013% of the Fe changes every day. The Fe metabolic rate may differ depending on the molecular species composing various cells. Assuming that myoglobin turnover takes place at the same rate as the average turnover, the total amount of myoglobin Fe turnover should be 41.6 μg (750 nmol) per day; this means 4.5×10^{17} atoms Fe per day. At least this amount of Fe must be collected by the motor neurons present in the spinal cord to provide Fe for muscle. The number of motor neurons in one spinal cord segment differs depending on the segment and ranges from 1500 to 70000 (Craw 1928; Tomlinson et al. 1973; Tomlinson and Irving 1977; Tsukagoshi et al. 1979). According to Sirkin and Kuhlenbeck (1966) the number of motor neurons present in the 31 segments comprising the whole spinal cord is estimated to range from 80000 to 160000.

Since the exact number of TfR present on the cell surface of a neuron is not known, let us assume that it is 5×10^4 per cell, which is derived from the TfR number of dividing cancer cells. The amount of Fe taken up is 36 atoms/TfR per hour (Iacopetta and Morgan 1983a), and the number of motor neurons is 2×10^5. Thus Fe taken up by all motor neurons is 8.7×10^{12} atoms per day. This means that motor neurons collect 2×10^{-5} of the Fe that may be required. In other words, if Fe is provided only by neurons for muscles, the turnover rate of Fe of myoglobin should be at least 5×10^4 times less than the average Fe turnover. Although there still remains a possibility that Fe is provided by Schwann's cells, we lack the data to discuss this possibility.

Similar calculations elucidate that the motor neurons as a whole should release Tf protein as a vehicle of Fe at the rate of 2.25×10^{17} molecules per day (about 30 mg per day). This is equivalent to about 125 times the amount of protein contained in the whole motor neurons.

These calculations are based on many assumptions and may not give accurate values. However, they suggest intuitively that it would be very difficult for motor neurons to provide a sufficient amount of Fe for muscle fibers unless the Fe turnover rate of muscle were much lower than the average rate in an entire body.

5.1.2 Presence and Release of Tf from Nerve Endings

If the motor neurons can synthesize Tf and collect Fe in sufficient amount, Tf must be transported on axonal flow, accumulated at the nerve terminal, and released to synaptic clefts which Tf traverses to reach the muscle fibers. Presumably, Tf may be released by electrical stimulation. The period required for donating a sufficient amount of Tf would be about 2 h, judging from the nerve stimulation to prevent muscle atrophy as described above (Eccles 1941, see Sect. 3.2).

Even if Tf is extracted from peripheral nervous tissue, this does not necessarily mean that Tf is derived from axon. It may be derived from Schwann's cells which construct a myelin sheath or other tissues which attach to axons. In this context, it is interesting that oligodendroglia which construct the myelin sheath in the central nervous system have Tf and its mRNA, suggesting that Tf is synthesized there (Bloch et al. 1985). In myelin-deficient mutant rats, no-Tf-positive oligodendocytes are observed immunohistochemically (Connor et al. 1987). Oh et al. (1981) stained axon with anti-sciatin antibody and claimed that Tf is transported on axoplasmic flow and released by electrical stimulation. However, their experiments contain the drawbacks pointed out in Sect. 4.4. They have not published papers on the matter of axoplasmic flow and release of Tf except for a brief description in a collection of proceedings (Oh and Markelonis 1982b), and details are not clear enough for further discussion. There has also been no report of whether Tf is stored at the nerve terminal.

If Tf is released from the nerve terminal, can it work effectively? In comparison, let us briefly consider ACh. As a neurotransmitter, the concentration of ACh at the synaptic cleft must be very low in the resting state. On excitation, the ACh released increases the ACh concentration so that it depolarizes the endplate. Then, ACh is inactivated by AChE, and its concentration quickly returns to a resting level. The concentration of Tf at the synaptic cleft in the resting state is not known but may be similar to Tf concentration of intercellular fluid, which is discussed below. And the concentration is probably

enough for Tf to bind with TfR at almost a saturation level. How does this additional Tf released from nerve terminal act?

5.1.3 Responses of Muscle Cells to Tf

If Tf is the substance which conveys the neurogenic trophic signal to muscle fibers, it is reasonable to assume that Tf is taken up solely at the synaptic region. Since denervation gives rise to atrophy of muscle fibers. Thus, Tf would not be provided from other parts of muscle fiber. Thus, TfR should not be found at other loci on muscle fiber than the synaptic region. However, immunohistochemical study showed that TfR is distributed throughout the whole surface of muscle fiber. Furthermore, numerous MVBs were found randomly scattered (Jefferies et al. 1985; Y. Gotoh et al., unpublished data).

One of the most clear-cut experiments to determine whether a substance is a neurogenic trophic factor is to examine its effect when administered to denervated muscles. Davis and Heinicke (1984) failed to prevent the denervation effect by administration of Tf. This result must be considered with caution, however, since it is very difficult to keep the substance at a desired concentration and a desired locus; this is because Tf injected into muscle is easily taken away by the circulation of tissue fluid and blood (K. Hayashi, personal communication).

Cell culture is frequently adopted in place of in vivo experiments, which are sometimes very difficult for studying the effect of trophic factor. The effects of Tf have been studied in cell culture on myoblasts and myotubes without innervation. This may not be a model of denervated mature muscle fibers, but serious consideration is required to adopt cultured cells as a model of denervation of embryonic and newborn muscle. Myogenic cell proliferation and growth take place without innervation in vivo, although the extent is limited (Zelena 1962; Butler et al. 1982). Cultured myogenic cells might rather be a model of cell growth in infantile animals without innervation than a model of denervated mature muscle fibers.

It may be important, however, to compare the effects of denervation in vivo and Tf removal in vitro. The respective effects of these on protein synthesis is not necessarily equal, as described in Sects. 2.2 and 5.2. Denervation gives rise to activation of Ca-activated neutral proteinase (Elce et al. 1983; Hussain et al. 1987), suggesting the promotion of protein degradation. This enzyme is inhibited by proteinase inhibitors such as E64 and leupeptin, although it is not known whether these inhibitors prevent muscle atrophy due to denervation. On the other hand, Tf removal does not necessarily result in the stimulation of protein degradation (Markelonis et al. 1980; Shoji and Ozawa 1986). E64 does not prevent a myotube from atrophy following Tf removal (Y. Hagiwara, unpublished data).

As mentioned above, there is yet scarce evidence to show that Tf is a muscle trophic factor which is released from nerve terminals to maintain muscle fibers. Although Oh et al. (1988) claim that "Tf may serve as a useful model substance for further elucidation of neural influence on myogenesis and maintenance of adult muscle," it is difficult to concur with this claim. The role of this "sciatin concept," however, must be seen as having served as a trigger for probing the aspects of a neurogenic trophic factor in detail.

5.2 Tf as a Humoral Trophic Factor

In considering a humoral trophic factor, the same logic is used as in considering a neurogenic trophic factor. In a neurogenic factor, a synthesized substance is accumulated at the nerve terminal and released to traverse the synaptic cleft, a distance only about 500 Å. In a humoral factor, the distance from the organ to the target is very long, spanning from the liver to muscles in this case.

5.2.1 Synthesis, Presence, and Release of Tf

Tf protein is synthesized in the liver and released into the bloodstream. Since liver contains large amount of ferritin, it is possible that holoTf is released. If apoprotein is released, it can bind Fe in the intestine where Fe is absorbed (for review see Bezkorovainy 1980). Blood plasma contains about 2.5 mg/ml Tf. In mammals, Fe saturation is about 40%, and in avians nearly 90% (Kimura et al. 1982; T. Hasegawa, unpublished data). Since blood does not make direct contact with muscle fibers, Tf would have to pass through the capillary wall to enter the tissue fluid; no study on the feasibility of Tf passing through muscle capillary wall has yet been made. But ferritin (molecular mass, 480 kDa; molecular diameter, about 110 Å) and myoglobin (molecular mass, 17.8 kDa; molecular size, 25×34×42 Å) can pass through it. It is known that permeability depends on molecular size (Bruns and Palade 1968; Simionescu et al. 1973). Lf (molecular mass, 80 kDa; a dimer of ellipsoid with molecular size, 55×35×33 Å; Anderson et al. 1987) stands between ferritin and myoglobin. The size of Tf is very close to Lf. Therefore, it is reasonable to assume that Tf can pass through capillary wall and enter the tissue fluid.

5.2.2 Tf in Tissue Fluid

The actual concentration of Tf in muscle tissue fluid is not known and may vary depending on the tissue or locus. Tf concentration ranges, for example, from 9 to 25 μg/ml in cerebrospinal fluid (Bleijenberg et al. 1971). It is noteworthy that these concentrations are high enough to stimulate myogenic cell growth in vitro (Shimo-Oka et al. 1986).

The presence of a considerable amount of Tf in muscle tissue fluid is suggested by the findings that muscle tissue extract stimulates myogenic cell growth when added to culture medium, and that Tf in intracellular space is clearly stained immunohistochemically (Matsuda et al. 1984b). Tf in tissue fluid must be internalized into muscle fiber, and apoTf must be ejected into tissue fluid. Since molecular change does not occur during this cycle (Kimura et al. 1985), there must be a decrease in holoTf and an increase in apoTf in tissue fluid if there is no reservoir of holoTf.

Another problem is whether blood can supply the tissue fluid with a sufficient amount of holoTf for Tf exchange. Extracellular fluid including blood and tissue fluid ranges from 11 to 18 kg in men and from 8.5 to 13 kg in women. Plasma volume is 2.56 l per 75 kg in men and 2.01 l per 55 kg in women (Jandl 1987). Thus, extracellular volume is 2.5–5 times larger than plasma volume. If Tf concentration in tissue fluid is assumed to be 20 μg/ml, the total amount of plasma Tf is about 20 times greater than that of tissue fluid Tf since plasma Tf concentration is more than 100 times higher than that of tissue fluid. Therefore, blood can safely serve as a reservoir of holoTf (see Sect. 3.1). In this system, Tf recycles from blood to cells and from cells to blood; therefore, as a vehicle of FeTf it may not be deficient. Fe may be continuously provided to apoTf from the intestine, liver, etc.

5.2.3 Tf Uptake System in Muscle Cells

TfR is present in myoblasts, myotubes, and muscle fibers (Shimo-Oka et al. 1986; Tsavaler et al. 1986; Stamatos and Fine 1986; Sorokin et al. 1987; Hasegawa and Ozawa 1982; Jefferies et al. 1985; Oh et al. 1988). TfR is not restricted to the neuromuscular junction but is widely distributed throughout the entire length of the muscle fibers. TfR is present not only on the muscle fiber cell surface but also in cytoplasm and is especially notable in fibers with well-developed mitochondria like red muscle fibers (Y. Gotoh et al., unpublished data). This findings is compatible with the finding that Tf in the intercellular space in chicken red muscles is more abundant than that in white muscles (Matsuda et al. 1984a, b).

There is no animal experimental study to show that Tf added to blood is actually taken up by muscle cells. Such a study is very difficult unless an animal lacking Tf can be used since the extrinsic Tf that is administered is diluted by intrinsic Tf. Tf-deficient animals, if indeed there are any, may die before they are born since such animals would not grow or survive. There is, however, one human case known in which the serum Tf level was severely decreased at a certain period in the boyhood of the patient. The boy visited a hospital with symptoms of severe anemia and was diagnosed as having Tf deficiency, a very rare disease. He was administered apoTf at certain intervals to improve the anemia to a point at which it was at a level for everyday life. The amount of

Tf given was far less than naturally occurring Tf in a normal individual. For an unknown reason, the concentration of Tf in his serum increased spontaneously and the anemia was cured. During this period, changes in body length and weight were recorded. While serum Tf was low, growth was severely retarded. He started growing again as serum Tf increased and finally reached 10% of the normal level. During the anemic period, doctors made no muscle examination and were not aware of a denervation-like atrophy (Hayashi 1984, and personal communication). However, it is clear his muscles did not grow during this period, because his entire body did not grow.

This case does not answer all the questions we have, but if does show that Tf works as a growth-promoting factor not only in vitro but also in vivo.

6 Summary and Conclusion

Muscle cells grow by proliferation and protein accumulation. During the initial stages of development the participation of nerves is not always required. Myoblasts and satellite cells proliferate, fusing to form myotubes which further differentiate to muscle fibers. Myotubes and muscle fibers grow by protein accumulation and fusion with other myogenic cells. Muscle fibers finally reach a quasi-steady state which is then maintained for a long period. The mechanism of maintenance is not well understood. However, it is clear that protein metabolism plays a paramount role. The role played by satellite cells in the maintenance of muscle fibers is not known.

Growth and maintenance of muscle cells are under the influence of various tissues and substances. Among them are Tf and the motor nerve, the former being the main object of this review and essential for both DNA and protein synthesis.

Two sources of Tf have been proposed, i.e., the motor nerve and the tissue fluid. The first proposal is that the nervous trophic influence on muscle cells is mediated by Tf which is released from the nerve terminals. In this model, the sole source of Tf which is donated to muscle cells should be the nerve, and Tf should not be provided for muscle fiber at sites other than the synaptic region; otherwise, denervation atrophy would not occur, since Tf provided from TfR located at another site would cancel the effect of denervation. The second proposal is that Tf is provided from tissue fluid. This implies that an adequate amount of Tf is transferred from serum to tissue fluid; in this case TfR may be distributed over the entire surface of the cells.

The trophic effects of the motor neuron have been studied in vivo, but its effects of myoblast proliferation have not been determined. There are few experiments on its effects on myotubes. Most work has been made on muscle fibers, where innervation is absolutely required for their maintenance. Without it, muscle fibers atrophy, although they do not degenerate.

In contrast, almost all the work on Tf has been performed in vitro. Its effects on myoblast proliferation and myotube growth and maintenance have been established; myotubes degenerate following Tf removal. But its effects on mature muscle fibers in vivo are not well understood. Muscle fibers possess TfR all over on their cell surface and contain a variety of Fe-binding proteins, such as myoglobin. It is entirely plausible that muscle fibers require an amount of Tf, and that this is provided by TfR scattered on the cell surface.

The key to the problem on the source of Tf is to clarify whether a single motor neuron can provide FeTf sufficient for a group of muscle fibers which are under the control of that neuron. Such provision seems extremely difficult in regard to both Fe and Tf protein. Therefore, the present evidence does not support the first proposal, that Tf is provided by the motor nerve.

Tf is synthesized in the liver and secreted into blood which contains it in sufficient amount and can serve as a reservoir of tissue fluid. Since TfR is distributed widely on the muscle cell surface, the cells can take up Tf from the tissue fluid. Therefore, the second proposal is feasible.

Fe transported by Tf is required for muscle cell proliferation, growth, and maintenance. Without its sufficient provision, cell proliferation and protein synthesis, and thus differentiation, of muscle cells cease. Further, myotubes degenerate and finally die out. Muscle cells in vivo are constantly immersed in tissue fluid containing Tf, and Tf deficiency is unlikely under physiological conditions. Therefore, muscle fibers atrophy following denervation apparently in spite of the presence of Tf. Provision of Tf and motor nerve innervation are both essential for the growth and maintenance of muscle cells. The trophic mediator from the motor neuron to muscle fibers must be different from Tf. Existence of such a specific factor in nerve other than ACh is still not known.

Cell culture is widely used for studying various growth factors and is acknowledged to be a very important method. However, great caution must be given to superimposing the in vitro phenomena on the in vivo phenomena in the case of muscle cells. Myotubes are innvervated in vivo and further differentiate into muscle fibers, while myotubes in vitro are not under the control of nerves in most experiments on growth factors and do not further differentiate. It is easy to observe the effects of Tf on myogenic cells, but it is not easy to conclude whether such effects can be observed in vivo. Nevertheless, muscle cell culture has been as useful tool for studying the effect of Tf on growth and differentiation in the absence of nervous effects.

In considering the trophic phenomena of muscle cells, various humoral factors which are provided by the tissue fluid have been rather ignored. Indeed, experiments on these humoral factors are not easy in vivo, and, as described, studies with cultured cells are indispensable despite their inherent difficulties. It cannot be too strongly emphasized, however, that attention should always be focused on the effects of these humoral factors in addition to those of the motor nerves on myogenesis and muscle fiber maintenance.

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Subject Index