A UBIQUITOUS CALCIUM BINDING PROTEIN (CALMODULIN) AND ITS POSSIBLE PHYSIOLOGICAL FUNCTION

BY

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"Science began in the distant past, long before human history was being recorded. Its mother was tribal magic. The same mother gave birth to religion and probably even earlier, to art. Thus science, religion, and art have always been blood brothers. Their methods differ, but their aim is the same: to understand and interpret the universe and its workings and, from this, to promote the material and spiritual welfare of man where possible".

Paul B. Weisz, The Science of Biology, McGraw-Hill, 1963.

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ABSTRACT

This study has centered on the following aspects of the protein modulator: (1) the phylogenetic distribution of the protein modulator, (2) a comparison of the physical-chemical properties of protein modulator from a variety of crude animal extracts,(3) comparison of the physical, Ca^{2+} binding, and chemical properties of a purified primitive protein modulator (earthworm), with the purified protein modulator from bovine heart, (4) analysis of the physiological significance of protein modulator in rabbit skeletal muscle.

An examination of crude extracts from animals representative of the major phyla has revealed that the protein modulator has a ubiquitous phylogenetic distribution. This initial observation has been extended to the vascular plants. Since the protein modulator from crude plant and animal extracts was capable of the activation of mammalian cyclic nucleotide phosphodiesterase, and because polyacrylamide gel electrophoretic analysis of the crude animal extracts has revealed a single band of protein modulator activity of similar relative mobility, it is proposed that the functional as well as the physical-chemical properties of the protein modulator have been highly conserved during evolution.

A comparison between purified protein modulator from the earthworm Lumbricus terrestris and from bovine heart has revealed many similar properties. Both proteins have molecular weights of approximately 18,000 daltons, isoelectric points of about pH 4, similar and characteristic ultraviolet spectra, and similar amino acid compositions. Both proteins bind calcium ions with high affinity. However, the protein modulator from Lumbricus terrestris binds 2 mol of Ca^{2+} ions with equal affinity K_{diss} 6 μM whereas the protein modulator from bovine heart binds 4 mol of Ca^{2+} ions with differing affinities. Tryptic peptide maps of both protein modulators show identity in six of twenty peptides therefore suggesting homology, but clearly demonstrating non identity. The similarity of the protein modulator from Lumbricus terrestris and bovine heart has further supported the postulate that the protein modulator represents an universal and highly conserved protein.

The possible physiological function of protein modulator in rabbit skeletal muscle, has been investigated. The demonstration of the regulation of phosphorylase kinase, myosin light chain kinase, and an uncharacterized glycogen synthase kinase by Ca^{2+} and the protein modulator has suggested that in rabbit skeletal muscle the protein modulator serves to regulate glycogen metabolism and myosin light chain phosphorylation.

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EXPERIMENTAL RESULTS

LIST OF ABBREVIATIONS

CD	Circular dichroism
DEAE	Diethylaminoethyl
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylenediamine-N, N'-tetraacetic acid
EGTA	Ethylene glycol bis(β-aminaethyl ether)-N, N'-tetraacetic acid
ELC	Essential light chains
ICBP	Intestinal calcium binding protein
MDPK	Modulator dependent protein kinase
PDE	Cyclic nucleotide phosphodiesterase
PM	Protein modulator
RLC	Regulatory light chains
SDS	Sodium dodecyl sulfate
TRIS	Tris (hydroxymethyl) aminomethane
TN-C	Troponin-C
TN-I	Troponin-I
TN-T	Troponin-T

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INTRODUCTION

Ca^{2+} has been implicated in a great diversity of cellular functions including stimulus-secretion coupling, glycogenolysis, gluconeogenesis in liver and kidney cells, and stimulus-contraction coupling in all forms of muscle. Based on these observations Ca^{2+} has been proposed to be a universal second messenger and suggested not only to function within a variety of tissues within a single organism, but also in many different organisms (Rasmussen et al, 1972).

Since the principal if not exclusive mediators of the intracellular actions of the second messengers c-AMP and c-GMP have been identified as protein kinases much attention has been directed toward the elucidation of the intracellular mediators of the actions of Ca^{2+}. One mediator has recently been identified as the protein modulator, and the mechanism by which enzyme activity can be regulated by the protein modulator and Ca^{2+}, is as follows (Wang et al, 1975).

$$Ca^{2+} + PM \rightleftharpoons Ca^{2+} - PM \rightleftharpoons Ca^{2+} - PM^*$$

$$PM^* - Ca^{2+} + E \rightleftharpoons E - PM^* - Ca^{2+} \rightleftharpoons E^+ - PM^* - Ca^{2+}$$

where the symbols denote protein modulator (PM), regulated enzyme (E), and conformational changes (* +).

The model proposes a central role for the protein modulator as a Ca^{2+} receptor. In the absence of Ca^{2+} the protein modulator is inactive, however after binding Ca^{2+} the protein modulator is capable of stoichiometric interaction with the regulated enzyme. The importance of protein modulator mediated Ca^{2+} regulation of enzyme activity is best illustrated by the impressive number of enzymes regulated by this mechanism. These enzymes include cyclic nucleotide phosphodiesterase, brain adenylate cyclase, erythrocyte (Ca^{2+} - Mg^{2+}) ATPase, myosin light chain kinase, phosphorylase kinase, and synaptosomal membrane protein kinase. While Ca^{2+} has been implicated as a second messenger in many different organisms, the possibility that the protein modulator may function as a Ca^{2+} receptor in these organisms has not been investigated.

This thesis is dedicated in part to the elucidation of the phylogenetic significance of protein mediated Ca^{2+}-linked regulation of enzyme activity, and in part to the elucidation of the physiological functions of protein modulator in rabbit skeletal muscle.

Three experimental approaches were used to investigate the phylogenetic significance of protein modulator mediated Ca^{2+} regulation. The first approach involved the examination of crude plant and animal extracts for the

presence of protein modulator. The Ca^{2+}-dependent activation of bovine heart phosphodiesterase by extracts was used to determine protein modulator activity. For the second approach the relative mobility of protein modulator activity of the extracts was examined by polyacrylamide gel electrophoresis, which provided a means of examining possible evolutionary changes in the physical-chemical properties of the extract modulator. Third, an in depth analysis of the physical, chemical and Ca^{2+}-binding properties of a purified protein modulator from an invertebrate was undertaken, and a comparison between purified protein modulator from this invertebrate and from bovine heart allowed a more detailed evaluation of any evolutionary changes of the protein modulator.

After investigation of the phylogenetic significance of protein modulator mediated Ca^{2+} regulation it was decided to investigate the physiological function of protein modulator mediated Ca^{2+} regulation in rabbit skeletal muscle. Rabbit skeletal muscle was chosen because of the well established regulatory role of Ca^{2+} in that tissue. While Ca^{2+} has been demonstrated to coordinate muscle contraction, myosin light chain phosphorylation, and glycogenolysis in this tissue, a possible role for the protein modulator has not been investigated. To this end, the possible regulation of myosin light chain phosphorylation and glycogen metabolism by Ca^{2+} and the protein modulator has been studied.

The results of this study have allowed construction of a model outlining the possible role of protein modulator in rabbit skeletal muscle.

I. ISOLATION AND CHARACTERIZATION OF THE PROTEIN MODULATOR

1. Discovery of the Protein Modulator

The discovery of the protein modulator was actually as a result of investigations into the properties of cyclic nucleotide phosphodiesterase (EC 3.1.4.17). Cheung (1967) reported that during purification of phosphodiesterase from bovine brain cerebrum a substantial loss of phosphodiesterase activity occurred. Cheung suggested as a possible explanation for the inactivation of phosphodiesterase during purification that "a stimulatory factor present with the enzyme must have been dissociated during the course of its purification". This suggestion was based on two observations, the presence of a non-dialyzable substance obtained from the brain extract capable of activating the otherwise inactive partially purified phosphodiesterase, and second, the calculation that the activity of a mixture of crude and partially purified enzyme was greater than the sum of the activities of the two enzymes assayed separately. Further evidence was provided when it was discovered that chromatography of crude phosphodiesterase on DEAE-cellulose resulted in a loss of enzyme activity which could be reconstituted by addition of fractions eluted after the peak activity of phosphodiesterase (Cheung, 1969; Cheung, 1970). The phosphodiesterase activating factor was characterized as a

protein possessing neither proteolytic nor phosphodiesterase activity. The activating factor was reportedly stable to heat, acid pH, and 8M urea. Stimulation of the purified enzyme by the activator appeared specific, as several other proteins of various molecular weights were unable to mimic its stimulatory effect. Stimulation was independent of the time of preliminary incubation with the protein activating factor, but dependent on its concentration in the reaction mixture, suggesting a stoichiometric interaction between the protein activating factor and phosphodiesterase (Cheung, 1971).

A similar heat stable non-dialyzable protein activating factor was reported in bovine heart by Goren and Rosen (1971). The bovine heart phosphodiesterase and the protein activating factor were separated by DEAE-cellulose chromatography.

Kakiuchi and coworkers showed that phosphodiesterase activity in the 100,000 x g supernatant of a rat brain homogenate was stimulated by micromolar concentrations of Ca^{2+} in the presence of Mg^{2+} (Kakiuchi and Yamazaki, 1970a). Further results suggested that the stimulatory effect of Ca^{2+} on the crude enzyme was enhanced by the addition of a non-dialyzable and thermostable factor present in the brain extract (Kakiuchi and Yamazaki, 1970b). Gel filtration of rat cerebral supernatant fluid (EGTA was not added

to buffers) resolved two peaks of phosphodiesterase activity. The high molecular weight fraction (150,000 daltons) was named Ca^{2+}/Mg^{2+}-dependent cyclic nucleotide phosphodiesterase because of the stimulatory effect of Ca^{2+} and the protein activating factor (Kakiuchi et al, 1971). The stimulation of enzyme activity by Ca^{2+} was suggested to be completely dependent on the presence of the protein activating factor (Kakiuchi et al, 1973). The effect of the activating factor on enzyme activity was observed when the concentration of Ca^{2+} in the medium was above a threshold value (2 μM).

2. Species and Tissue Distribution

The protein modulator has been demonstrated in all mammalian tissues examined, which include bovine brain (Cheung, 1970), cardiac muscle (Goren and Rosen, 1971) rat brain (Kakiuchi and Yamazaki, 1970b) rat adrenal, kidney, epididymal fat pad, bone marrow, liver, testes, thymus, human blood platelet, brain (Smoake et al, 1974) uterus (Smoake et al, 1974; Kroeger et al, 1976), rat anterior pituitary (Russel and Thorn, 1977) and porcine coronary arteries (Wells et al, 1975). Since protein modulator from human, porcine, and rat brain and bovine heart cross-activated partially purified phosphodiesterase from the same tissues, Cheung (1971) has suggested that the protein

modulator lacks species specificity. This suggestion has been confirmed and extended by Waisman et al, (1975) who have examined a number of invertebrates and higher plants for protein modulator activity; in all species examined protein modulator activity (assayed with bovine heart phosphodiesterase) was demonstrated.

The distribution of the protein modulator has been quantitated in eight different rat tissues by Smoake et al, (1974). The rat testes contained the highest specific activity. Phosphodiesterase activity did not appear to correlate with modulator activity, for example rat testes was the richest source of protein modulator but one of the poorest sources of phosphodiesterase. Waisman et al, (1975) have also suggested a non parallel distribution of protein modulator and phosphodiesterase activities. Crude extracts prepared from a number of invertebrates were found to be rich sources of protein modulator but to contain little if any Ca^{2+}-activated phosphodiesterase activity. Similar results were documented for the higher plants. The results (Waisman et al, 1975) have suggested that unlike the protein modulator, Ca^{2+}-activated phosphodiesterase may have a limited phylogenetic distribution. It therefore appears that protein modulator and phosphodiesterase activities have non parallel tissue and phylogenetic distribution.

3. Subcellular Distribution

The subcellular distribution of the protein modulator has been determined in human blood platelets and liver parenchymal cells (Smoake et al, 1974), and bovine brain cortex (Cheung et al, 1975b). The majority of the protein modulator activity was found in the cytosol although small but significant modulator activity was reported in nuclear and microsomal preparations. Drabikowski et al, (1977b) have analyzed several tissues for the subcellular distribution of protein modulator, and found protein modulator activity to be present in both the 100,000 x g supernatant and pellet of skeletal muscle (rabbit), cardiac muscle (bovine), smooth muscle (rabbit uterus and chicken gizzard), adrenal medulla (bovine), brain (bovine), and platelets (bovine). Gnegy et al (1976) have demonstrated the presence of protein modulator in the washed 105,000 x g pellet of rat brain and adrenal medulla. In the subcellular particulate fractions of rat brain the concentration of modulator was highest in the microsomal fraction followed by the mitochondrial and nuclear fractions. Gradient centrifugation of the main mitochondria subfraction revealed that the modulator was concentrated in those fractions containing mainly synaptic membranes (Gnegy et al, 1977). The presence of protein modulator has also been reported by Schulman

and Greengard, (1978) in synaptosomal preparations.

These studies suggest a partly soluble, partly particulate subcellular distribution for the protein modulator.

4. Ontogenetic Development

Smoake et al, (1974) and Strada et al (1974) have examined the ontogenetic development of protein modulator in a variety of rat tissues. The ontogenetic development was found to be characteristic of the particular tissue examined. In general, it was observed that only minor changes in protein modulator activity occurred during ontogenetic development in many of the tissues examined (rat brain, thymus, and liver). In contrast, about a three fold increase in protein modulator activity was reported in testes. In none of the tissues examined was the development of protein modulator and phosphodiesterase activity parallel. For example, the Ca^{2+}-activated phosphodiesterase activity in rat cerebrum was found to increase twenty fold, whereas the protein modulator activity remained about the same (Strada et al, 1974).

5. Physical Properties

The protein modulator has been purified to homogeneity from bovine heart (Teo et al, 1973), bovine brain (Lin et al, 1974), porcine brain (Teshima and Kakiuchi, 1974;

Wolff and Brostrom, 1974; Klee, 1977a) rat testes (Beale et al, 1977) porcine and adrenal medulla (Egrie and Siegel, 1975) blood platelets (Muszbek et al, 1977) chicken gizzard (Dabrowski et al, 1977) electroplax of Electrophorus electricus (Childers and Siegel, 1975), and the earthworm (Waisman et al,1978a). Wolff and Siegel, (1972) purified a Ca^{2+}-binding phosphoprotein from porcine brain and from bovine adrenal medulla and testes (Brooks and Siegel, 1973a, b). This protein was later identified as the protein modulator (Wolff and Brostrom, 1974).

The protein modulator from a variety of sources has been extensively characterized by several groups of researchers. In general, all modulators examined exhibit very similar physical and chemical properties. Molecular weight determination by hydrodynamic methods have suggested a range of molecular weight from 15,000 to 19,000 daltons; these discrepancies appear to depend more on the method of determination than the source of the modulator. Watterson et al, (1976) have shown that determination of modulator molecular weight by sedimentation equilibrium method in low ionic solution results in low values. Overestimation of the molecular weight of modulator on gel filtration columns has suggested anomalous behavior of the protein modulator on gel filtration columns (molecular weight estimated by this method has been determined as 28,000 daltons, (Teo et al,

1973). The molecular weight of the bovine brain protein modulator has been determined from the amino acid sequence to be 16,723 daltons (Vanaman et al, 1977).

The protein modulator is very acidic, the isoelectric point has been determined to be about 4.0. The modulator has an atypical ultraviolet spectrum. Instead of having an absorption maximum at 280 nm as do most globular proteins, considerable vibrational structure in the region of 250 - 280 nm, and absorption peaks at 253, 259, 265, 258, and 276 nm have been reported for the modulator by Wang et al, (1975), Stevens et al, (1976), Watterson et al, (1976), Liu and Cheung, (1976), Kuo and Coffee, (1976a), Klee (1977a), and Dabrowski et al, (1977a). The unique optical properties result from the presence of a high phenylalanine/tyrosine ratio (8:2) and an absence of tryptophan. Furthermore, the protein modulator has an unusually low absorptive index of about 2.0 at 275 nm (1% solution; Watterson et al, 1976; Stevens et al, 1976).

6. Amino Acid Composition

The amino acid compositions of protein modulator purified from bovine heart (Wang et al, 1975) bovine brain (Lin et al, 1974; Watterson et al, 1976), porcine brain (Wolff and Siegel, 1972; Klee, 1977a), bovine adrenal medulla (Kuo and Coffee, 1976), rat testes (Beale et al,

1977), electroplax of Electrophorus electricus (Childers and Siegel, 1975) and Lumbricus terrestris (Waisman et al, 1978a) are remarkably similar. The protein modulator has a high content of acidic residues and a relatively low content of basic residues, consistent with the observed isoelectric point of 4.0. Liu and Cheung, (1976) and Walsh and Stevens (1977) have demonstrated that more than 70% of the total number of acidic amino acids in the acid hydrolyzate are represented by glutamic and aspartic acid, less than 30% are represented by glutamine and asparagine. Cysteine is commonly reported absent, although one residue of cysteine has been reported for the earthworm (Waisman et al, 1978a) and bovine adrenal medulla (Kuo and Coffee, 1976a) protein modulator. Tryptophan is consistantly reported as absent.

The presence of one mol of unidentified ninhydrin-positive basic compound in the acid hydrolyzate of bovine brain protein modulator was first reported by Watterson et al (1976). This unusual amino acid has been identified as ε- N - trimethyllysine, and demonstrated in the hydrolysates of all species tested including bovine, porcine, rabbit, and chicken brain protein modulator (Vanaman et al, 1977; Miyake and Kakiuchi, 1977), and rat testes (Jackson et al, 1977).

7. Amino Acid Sequence

Determination of the amino terminal of the protein modulator has yielded conflicting results. The dansyl chloride procedure has been used to identify valine (Lin et al, 1974) or isoleucine (Kuo and Coffee, 1976) as the amino terminal. Watterson et al, (1976) failed to detect an amino terminal amino acid, using a sequenator and suggested that the protein modulator contained a blocked amino terminal. Vanaman et al, (1977) have sequenced the bovine brain protein modulator and determined that the amino terminus of the modulator was acetylated and consisted of Ac-met-asp-asp. The amino terminus of the rat testes protein modulator was identified by Dedman et al, (1977) as Ac-ala-asp-glu.

Sequence studies have confirmed that the protein modulator is a simple protein (Vanaman et al,1977). Earlier claims that the bovine heart modulator was a glycoprotein (Teo et al, 1973) and that the porcine brain protein modulator was a phosphoprotein may have been due to contaminants (Wang et al, 1975).

Stevens et al (1976)have demonstrated that tryptic peptide maps of bovine heart and bovine brain protein modulator are indistinguishable,and have suggested that these are the same protein. Earlier comparisons by

Brooks and Siegel (1973a, b) demonstrated that the protein modulator was essentially identical in all bovine tissues examined (brain, heart, adrenal medulla, and testes), according to gel electrophoretic comparisons. Furthermore, Brooks and Siegel (1973a) demonstrated that tryptic peptide maps of bovine brain and adrenal medulla were identical. The results suggest that within the bovine tissue the protein modulator might exist as a single protein entity.

The complete sequence of the modulator from bovine brain (Vanaman et al, 1977) and rat testes (Dedman et al, 1978) has been determined, and a comparison of these sequences has established that the two modulators are remarkably similar.

II. MECHANISM OF ACTIVATION OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE BY PROTEIN MODULATOR

1. Overview

The characteristics of enzyme activation by protein modulator have been studied in many laboratories, using partially purified Ca^{2+}-activatable phosphodiesterase largely free of protein modulator. One of the most important observations was that enzyme activation depends on the simultaneous presence of Ca^{2+} and the protein modulator (Teo and Wang, 1973; Kakiuchi et al, 1973; Lin et al, 1974; Wolff and Brostrom, 1974; Wickson et al,

1975) i.e. the enzyme is not activated by Ca^{2+} in the absence of protein modulator nor is the enzyme activated by protein modulator in the absence of Ca^{2+}. Two mechanisms could account for this observation; enzyme activation could be due to binding of Ca^{2+} and protein modulator to phosphodiesterase, or the binding of Ca^{2+} by the protein modulator could constitute the active complex. The demonstration that protein modulator is a Ca^{2+}-binding protein has suggested that the binding of Ca^{2+} by modulator is necessary for enzyme activation. However, the two mechanisms do not have to be mutually exclusive and a possible contribution by the former mechanism can only be evaluated by examination of phosphodiesterase for Ca^{2+} binding properties. Wang et al, (1975) have shown that no significant Ca^{2+} binding was detected (gel filtration method of Hummel and Dreyer, 1962) with an enzyme preparation approximately 20% pure, at a concentration as high as 2 mg/ml. This data support the hypothesis that Ca^{2+} activates phosphodiesterase by binding to protein modulator.

Since enzyme activation depends on the simultaneous presence of Ca^{2+} and protein modulator the apparent Ka for Ca^{2+} (concentration of Ca^{2+} required for 50% enzyme activation) depends on the amount of protein modulator

present in the reaction mixture. At saturating amounts of protein modulator an apparent Ka of 2.3 μM has been reported for the bovine heart enzyme and modulator (Teo and Wang, 1973). Other investigators using protein modulator from mammalian brain and from the earthworm have obtained similar Ka values of 2 - 8 μM (Kakiuchi et al, 1973; Lin et al, 1974; Wolff and Brostrom, 1974; Wickson et al, 1975; Waisman et al, 1978).

Mammalian phosphodiesterase depends on Mg^{2+} for activity, although this cation requirement can also be satisfied by Mn^{2+} (Butcher and Sutherland, 1962). The Ca^{2+}-activated phosphodiesterase has been shown to require millimolar concentrations of Mg^{2+} (Kakiuchi and Yamazaki, 1970a) for catalytic activity, and micromolar concentrations of Ca^{2+} for activation. Although at least one order of magnitude less potent, other ions such as Mn^{2+}, Sr^{2+}, Ba^{2+} and Co^{2+} may replace Ca^{2+} as an activating ion (Kakiuchi et al, 1972; Teo and Wang, 1973; Lin et al, 1974). Mn^{2+} is unique in that it can fulfill the metal requirements for both activity and activation.

Ca^{2+} has also been shown to be required for the association between the enzyme and protein modulator. Teshima and Kakiuchi (1974) demonstrated in a series of gel filtration experiments that an active PDE-PM complex

was formed in the presence of Ca^{2+}, but in the absence of Ca^{2+} this complex dissociates into two separate components, PDE and PM. The results suggest that the active enzyme-modulator complex is formed only in the presence of Ca^{2+}. Similar results have been reported by Lin et al, (1975) and Wickson et al, (1975).

Lin et al, (1975) suggested that the effect of Ca^{2+} on the formation of a PDE-MP complex was instantaneous. Cheung (1971) showed that enzyme activation was dependent on the modulator concentration but independent of pre-incubation of enzyme and modulator. Teo et al (1973) demonstrated that activation of phosphodiesterase by protein modulator could be decreased by dilution of the enzyme. Activation of phosphodiesterase by Ca^{2+} has been shown to be readily reversible (Lin et al, 1974; Wolff and Brostrom, 1974; Waisman et al, 1975). The modulator concentration required to activate phosphodiesterase has been demonstrated to depend on the c-AMP concentration (Wang et al, 1972; Teo et al, 1973). These results suggest a reversible interaction between the enzyme and protein modulator.

The results of studies of the interaction of protein modulator and phosphodiesterase has allowed formulation of a model of the mechanisms of activation of phosphodiesterase by Ca^{2+} and protein modulator (Teshima

and Kakiuchi, 1974; Liu et al, 1974; Wang et al, 1975). Essentially these models propose that activation of phosphodiesterase is a stepwise procedure. As a consequence of Ca^{2+} binding, the protein modulator undergoes a conformational change to an active conformation. This complex (Ca^{2+}-PM) is capable of association with phosphodiesterase to form a ternary complex (Ca^{2+}-PM-PDE). As a result of the formation of the ternary complex, phosphodiesterase undergoes a conformational change and an enhancement of enzyme activity occurs.

The individual steps of this mechanism are considered in detail in the following sections.

2. Calcium Binding Properties

Discrepancies exist in the literature regarding the number of classes of Ca^{2+} binding sites on the protein modulator and their relative affinities and capacities. Wolff and Siegel (1972) using ultrafiltration technique found that the bovine brain protein modulator bound one mol of Ca^{2+}/mol of protein with K_d 25 μM. Similarly Brooks and Siegel (1973b) found that the adrenal medulla modulator had a single site K_d 17 μM. Teo and Wang (1973), using a gel filtration technique found one high affinity site (K_d 2.9 μM) and three lower affinity sites (K_d 11.9 μM) for the bovine heart modulator. Lin et al (1974) using

equilibrium dialysis reported four Ca^{2+} binding sites, three of high affinity (K_d 3.5 μM) and a single low affinity site (K_d 18 μM) for the bovine brain protein modulator. Kuo and Coffee, (1976a) reported two Ca^{2+} binding sites of K_d 20 μM, using equilibrium dialysis. Watterson et al, (1976) reported that bovine brain modulator bound two mol of Ca^{2+} with high affinity (K_d 1 μM) and two mol with very low affinity (K_d 860 μM). Micro-scale equilibrium dialysis was used in this study. Waisman et al (1978a) using gel filtration technique reported that the earthworm modulator bound two mol of Ca^{2+} with K_d 6 μM. Dedman et al, (1977b) using equilibrium dialysis demonstrated four equivalent Ca^{2+} binding sites K_d 2.4 μM for the rat testes protein modulator. Wolff et al, (1977) has reported the existence of two classes of Ca^{2+} binding sites in the bovine brain modulator, one class bound three mol with K_d 0.2 μM and the other class bound one mol with K_d 1.0 μM. Binding studies were performed using equilibrium dialysis. Klee et al, (1977) observed two classes of Ca^{2+} binding sites, two high affinity sites (K_d 4 μM) and two low affinity sites (K_d 12 μM) using equilibrium dialysis of porcine brain protein modulator.

As pointed out by Wolff et al (1977) experimental determination of bound Ca^{2+} can be greatly affected by

trace contamination of buffers by Ca^{2+}. Variation in the concentration of Mg^{2+} in buffers from study to study could also produce confusing results. Dedman et al (1977b) have pointed out that ionic strength is an important factor when interpreting the effects of metal binding to protein modulator. It therefore stands to reason that many of the discrepancies reported in the literature could be composed of many factors including study to study variation in the concentration of Mg^{2+} and ionic strength of buffers, as well as trace contamination of Ca^{2+} in buffers, different methods of Ca^{2+} and protein concentration determination and denaturation of the protein modulator during the study.

A consensus of the reported literature suggests that the protein modulator binds a maximum of four mol Ca^{2+}/mol protein with μM dissociation constants when assayed at high ionic strength (> 40 mM). Concentrations of Mg^{2+} as high as 3 mM have no effect on this Ca^{2+} binding (in brain tissue free Mg^{2+} is believed to be about 1 mM, Veloso et al, 1973). While different classes of binding sites may be present there is no cooperativity in the binding of Ca^{2+} to protein modulator (as witnessed from the linearity of Scatchard plots). At low ionic strength cation binding properties become more complicated and competitive between Ca^{2+} and Mg^{2+} for the binding sites

may occur (Wolff et al, 1977).

3. Ca^{2+} Induced Conformational Changes

Considerable evidence has accumulated to suggest that the protein modulator undergoes changes in conformation as a consequence of Ca^{2+} binding. Wang et al, (1975) reported that upon binding Ca^{2+} the modulator undergoes changes in several of its physical properties; the ultraviolet absorption spectrum shows a small decrease in absorption and the tyrosine fluorescence emission at 315 nm exhibits a 30% enhancement in intensity. Ca^{2+} has also been shown to stabilize the protein modulator against tryptic or chymotryptic inactivation (Ho et al, 1975; Liu and Cheung, 1976) and against thermal inactivation (Wang et al, 1975).

Several investigators have examined the Ca^{2+} induced conformational changes in α helical content of the modulator. Optical rotary dispersion measurements made by Liu and Cheung (1976) have suggested that the helical content of the bovine brain modulator increases from 39% (Ca^{2+}-free media) to 57% in the presence of saturating Ca^{2+}. Based on ultraviolet circular dichroism measurements, Kuo and Coffee (1976) have suggested that the bovine adrenal medulla protein modulator undergoes a change in helical content from 20% to 40% in the presence of

saturating Ca^{2+}. Klee (1977) has estimated with far ultraviolet circular dichroism that the porcine brain modulator is composed of approximately 30 - 35% α helix, 50% random coil, and 15 - 20% pleated sheet. In the presence of Ca^{2+} a 5 - 8% increase in helical content and corresponding decrease in random coil occurs. Dedman et al (1977b) have also examined the far ultraviolet spectra of the modulator from rat testes, and calculated that the α helical content at 10^{-8} M Ca^{2+} was 45%. At 10 μM Ca^{2+} the α helical content increased to 54%. Similar measurements have been made by Wolff et al, (1977) who has determined that the α helical content of the bovine brain protein modulator changes from 28% in Ca^{2+} free media to 42% in the presence of saturating Ca^{2+}.

Kuo and Coffee (1976b) have observed an increase in the sedimentation coefficient of the adrenal medulla protein modulator, from 1.50S in the absence of Ca^{2+}, to 1.89S in the presence of Ca^{2+}. The Stokes radius determined on Sephadex G-100 was found to decrease from 21.9 ^{O}A in the absence of Ca^{2+} to 19.8 A^{O} in the presence of Ca^{2+}. From these studies Kuo and Coffee (1976b) concluded that removal of Ca^{2+} from the protein modulator results in a transformation from a compact symmetrical structure to one that is less ordered and more asymmetrical.

Wolff et al (1977) have examined the difference circular dichroism spectra of the bovine brain modulator in the presence and absence of Ca^{2+}. They reported that the difference spectra displayed maxima at 280, 269, 262, and 258 nm revealing contributions from both tyrosine and phenylalanine, and suggesting that the binding of Ca^{2+} modifies the environment of these residues. Similar results were reported by Klee (1976). Spectrophotometric titrations of the two tyrosine residues of the porcine brain protein modulator revealed that one tyrosine residue (pk'12) was buried in the hydrophobic core of the modulator and is not significantly affected by the conformational changes accompanying Ca^{2+} binding. The second tyrosine residue (pk' 10.4) is partially exposed to the solvent in the absence of Ca^{2+} and upon Ca^{2+} binding the pk' of this residue shifts to pk' 10.1 indicating increased exposure of this residue to the solvent. Changes in the environment of this residue are suspected to have resulted in the increased tyrosine fluorescence reported by Wang et al, (1975). Walsh and Stevens (1977) have reported that in the presence of Ca^{2+} the microenvironment of histidine may change. This result was based on the reactivity of the histidine residue to carboxymethylation in the presence and absence of Ca^{2+}.

In conclusion, it appears that Ca^{2+} binding causes

profound changes in the conformation of the protein modulator; α helical content is increased, the stokes radius decreases and the sedimentation coefficient increases suggesting that the protein modulator becomes a more compact symmetrical molecule. The microenvironment of one tyrosine, histidine, and one or more phenylalanine residues is altered by Ca^{2+} binding.

4. Ca^{2+} Dependent Stoichiometric Interactions of Protein Modulator and Phosphodiesterase.

The demonstration of the Ca^{2+}-binding properties of protein modulator (Teo and Wang, 1973), that both Ca^{2+} and protein modulator are required for enzyme activation (Teo and Wang, 1973; Kakiuchi et al, 1973), that phosphodiesterase does not bind Ca^{2+} (Wang et al, 1975), and that Ca^{2+} is required for the association of modulator and phosphodiesterase (Teshima and Kakiuchi, 1974) along with the reports of conformational changes in protein modulator induced by Ca^{2+} (Wang et al, 1975) has led to the suggestion that after Ca^{2+} binding the protein modulator undergoes a conformational change such that association with phosphodiesterase can subsequently occur (Wang et al, 1975).

That the Ca^{2+} binding to the protein modulator is related to enzyme activation is supported by the similarity between values reported for the apparent K_d of Ca^{2+} binding

and K_a of enzyme activation; 3 to 18 μM as compared to 2 to 8 μM (Teo and Wang, 1973; Lin et al, 1974). Klee (1977) has presented evidence that the bulk of conformational changes in the porcine brain protein modulator occur upon binding of the first two mol of Ca^{2+} (Klee reported two sites of Ca^{2+} binding of high affinity, K_d 4 μM and low affinity K_d 12 μM). Klee has suggested that the correspondence between the affinity of Ca^{2+} for the high affinity sites and the concentration required for half-maximal stimulation of phosphodiesterase (2 - 5 μM) further reinforces the idea that the high affinity sites rather than the low affinity sites are required for the formation of the ternary complex (Ca^{2+}-PM-PDE).

Dedman et al, (1977b) have examined the dependency on Ca^{2+} concentration of Ca^{2+} binding, conformational change (monitored by circular dichroism and tyrosine fluorescence) and phosphodiesterase activation. They concluded that the conformational changes induced by Ca^{2+} are required before protein modulator is capable of phosphodiesterase activation. In particular, approximately 80% of the tyrosine fluorescence enhancement and 95% of the α helical change occurred before the threshold activation of phosphodiesterase was obtained. Interestingly maximum phosphodiesterase activation corresponded to about 50%

Ca^{2+} binding (four equivalent sites in rat testes protein modulator reported by Dedman et al, 1977b).

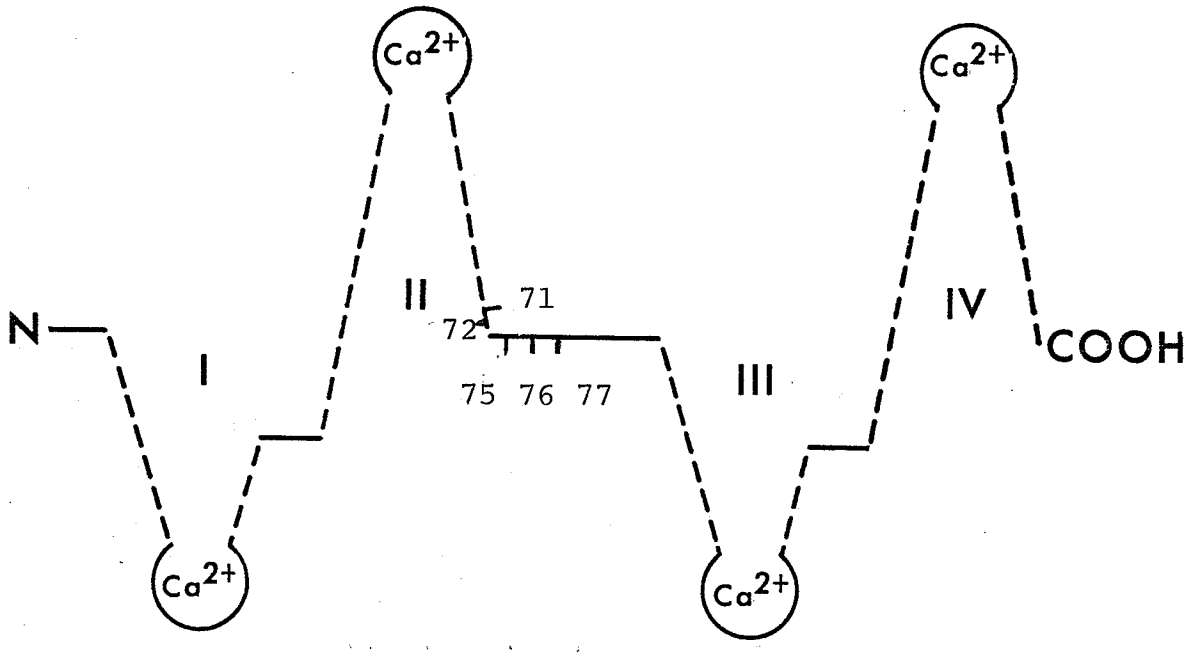
The stoichiometry of interaction between phosphodiesterase and protein modulator has been studied by Teshima and Kakiuchi (1974) and Lin et al, (1975) using gel filtration of partially purified phosphodiesterase. Values of 150,000 daltons for the free enzyme and 200,000 daltons for the enzyme modulator complex were obtained, however, the anomalous behavior of protein modulator during gel filtration has complicated interpretation of the results. Ho et al (1977) estimated a molecular weight (gel filtration) of 155,000 for the free enzyme and 230,000 for the modulator enzyme complex. The phosphodiesterase used in this study was 80% homogeneous. The results of the gel filtration experiments suggest that it is possible that more than one mol of modulator/mol enzyme may interact.

Dedman et al, (1977b) have determined the Hill coefficient of phosphodiesterase activation by increasing amounts of protein modulator is 2.0. This implies the interaction of multiple Ca^{2+}-PM complexes with a molecule of phosphodiesterase, however, impure phosphodiesterase was also used in this study. Determination of the exact stoichiometry of interaction of phosphodiesterase and protein modulator will have to await purification of phosphodiesterase.

Using the technique of chemical modification, Walsh (1978) has indicated that the site for interaction with phosphodiesterase is located between the second and third Ca^{2+}-binding regions of the protein modulator and is on the surface of the molecule. Walsh (1978) has also pointed out the importance of the integrity of both lysine and methionine residues for the expression of phosphodiesterase stimulating activity. Implicated in the interaction with phosphodiesterase was lysine 75 and 77 and methionine 71, 72, and 76. The position of these residues is illustrated in scheme I.

5. Protein Modulator Induced Conformational Changes in Phosphodiesterase.

The association between phosphodiesterase and protein modulator has been suggested to be accompanied by conformational changes in the enzyme. Wang et al, (1975) have determined that partially pure bovine heart phosphodiesterase is rapidly inactivated at 55^{o} when both Ca^{2+} and protein modulator are present, but is relatively stable in its free state. Other investigators have found similar results (Kakiuchi et al, 1975; Wolff and Brostrom, 1976; Liu and Cheung, 1976). The results suggest that the enzyme undergoes a conformational change upon association with the Ca^{2+}-PM complex. The failure of Ca^{2+} or protein



SCHEME I

DIAGRAMATIC REPRESENTATION OF THE STRUCTURE OF THE PROTEIN MODULATOR.

Dashed lines represent regions of α helix. The location of lysine 75 and 77 and methionine 71, 72, and 76 is indicated to show the possible site of interaction with phosphodiesterase (after Drabakowski et al, 1977a).

modulator alone to influence stability is in agreement with the observation that the enzyme does not interact with these ligands separately (Wang et al, 1975). That certain other activators (e.g. phospholipid) of phosphodiesterase also decrease the thermal stability of the enzyme (Wolff and Brostrom, 1976), the extent of this destabilization being identical to that produced by Ca^{2+} and protein modulator supports the view that the conformational change in phosphodiesterase induced by the Ca^{2+}-PM complex, as manifested by the change in enzyme stability is associated with enzyme activation.

Ho et al, (1977) have reported that purified phosphodiesterase can be stabilized by Ca^{2+} and protein modulator. An 80% homogeneous preparation was used in this study. The result suggest that the enzyme stability may depend on many factors.

The metal binding properties of the Ca^{2+}-PM-PDE complex have not been studied so that possible conformational changes in the Ca^{2+}-PM complex after binding to phosphodiesterase cannot be evaluated at this time.

6. Mechanism of Activation

A model has been proposed to explain the activation of cyclic nucleotide phosphodiesterase (Teshima and Kakiuchi, 1974; Wolff and Brostrom, 1974; Wang et al, 1975) and

adenylate cyclase (Brostrom et al, 1975; Cheung et al, 1975). This model is presented as follows: -

$$Ca^{2+} + PM \rightleftharpoons Ca^{2+}\text{-}PM \rightleftharpoons Ca^{2+}\text{-}PM^{*}$$

$$Ca^{2+}\text{-}PM^{*} + E \rightleftharpoons Ca^{2+}\text{-}PM^{*}\text{-}E \rightleftharpoons Ca^{2+}\text{-}PM^{*}\text{-}E^{+}$$

where the symbols denote conformational change (*, +). The stoichiometry of interaction has not been established, the equation simply depicts the necessary interaction of the two proteins. A similar model has been proposed by Dedman et al, (1977b) to compensate for the possible involvement of more than one Ca^{2+}-PM complex.

Klee, (1978) and Wallace et al (1978) have purified phosphodiesterase and suggested that phosphodiesterase consists of two subunits and that the Ca^{2+}-PM complex activates the enzyme by interaction with a specific Ca^{2+}-PM binding subunit. Recently, a heat stable inhibitor protein has been discovered and purified by Sharma et al (1978a, b) shown to undergo Ca^{2+}-dependent association with the modulator protein. This protein, unlike phosphodiesterase is monomeric. Similarly, Waisman et al (1978) have reported a monomeric structure for myosin light chain kinase. It therefore seems more likely that proteins capable of specific interactions with protein modulator contain a common structural domain rather than a common subunit

which is specific for the protein modulator.

An alternative mechanism of enzyme activation by the protein modulator has been proposed for rabbit skeletal muscle phosphorylase kinase by Cohen et al, (1978). These investigators reported the presence of protein modulator in near stoichiometric amounts with the other three subunits of phosphorylase kinase. The protein modulator appeared to be tightly associated with phosphorylase kinase and could not be removed from the enzyme by gel filtration in the absence of Ca^{2+}. Sharma and Wirch (1979) have reported the presence of tightly bound protein modulator associated with bovine lung phosphodiesterase. This tightly bound protein modulator could not be dissociated from phosphodiesterase by DEAE-cellulose or gel filtration chromatography in the absence of Ca^{2+}. Therefore, a second mechanism for the protein modulator mediated Ca^{2+}-linked regulation of enzyme activity may exist for certain enzymes; the mechanisms is presented as follows:

$$Ca^{2+} + E\text{-}MP \rightleftharpoons E\text{-}MP\text{-}Ca^{2+} \rightleftharpoons E^{*}\text{-}{}^{*}_{MP}\text{-}Ca^{2+}$$

where E and PM stand for enzyme and the modulator protein and the asterisk indicated the activated state of the protein species (after Sharma and Wirch (1979).

III. THE FAMILY OF HOMOLOGOUS Ca^{2+}-BINDING PROTEINS

Comparisons of the amino acid sequences of pike parvalbumins (Frankenne et al, 1973), rabbit skeletal muscle troponin-C (Collins et al, 1973; 1977), bovine brain protein modulator (Vanaman et al, 1977), rabbit skeletal muscle DTNB light chain (Collins, 1976), rabbit skeletal muscle alkali light chain (Frank and Weeds, 1974) and the mammalian intestinal calcium binding protein (Hofmann et al, 1977) have suggested that these proteins are homologous and may have been derived from a common ancestral protein. Together the proteins comprise a family of homologous proteins, the structural and functional interrelationships of the members of this family are discussed.

1. Parvalbumins

Parvalbumins are small (12,000 daltons), highly antigenic, acidic, Ca^{2+} binding proteins found in skeletal muscle of vertebrates. The amino acid sequence of parvalbumins from pike (Frankenne et al, 1973), carp (Coffee and Bradshaw, 1973), frog (Capony et al, 1975), rabbit (Enfield et al, 1975; Capony et al, 1976) and hake (Capony et al, 1973) has been reported. The amino acid sequence and chemical data indicate that all known parvalbumins are isostructural with that of carp (Kretsinger, 1979).

Kretzinger and Nockolds, (1973) first reported that the internal sequence repeats of parvalbumin suggested the existence of three homologous regions, and therefore parvalbumin may have evolved by gene triplication of a primitive calcium binding unit termed the "EF hand". The crystal structure of the carp parvalbumin has been determined and refined at 1.9 ^{o}A resolution (Kretsinger et al, 1971; Moews and Kretsinger, 1975). Based on X-ray crystallography work, Moews and Kretsinger, (1975) demonstrated the existence of six α-helical regions, A through F. Of the non helical loops between α-helices A and B, C and D, and E and F only the loops between C and D, and E and F bind Ca^{2+}. The Ca^{2+} ions are ligated to oxygen atoms donated by six amino acids, four of which are acidic. Each Ca^{2+} binding site lies in a pocket between two helices, and the structure is stabilized by the packing of apolar residues, present within these helices into a hydrophobic core. Helix E, loop EF, and helix F represent the basic structural and homologous domain, called the EF region (hand). The AB and CD regions are homologous to the EF region, although Kretsinger (1978) has pointed out that the AB region is barely recognized as a homolog. Each EF region contains about 33 residues. The Ca^{2+}-binding loops between the regions of α-helix are in β-antiparallel

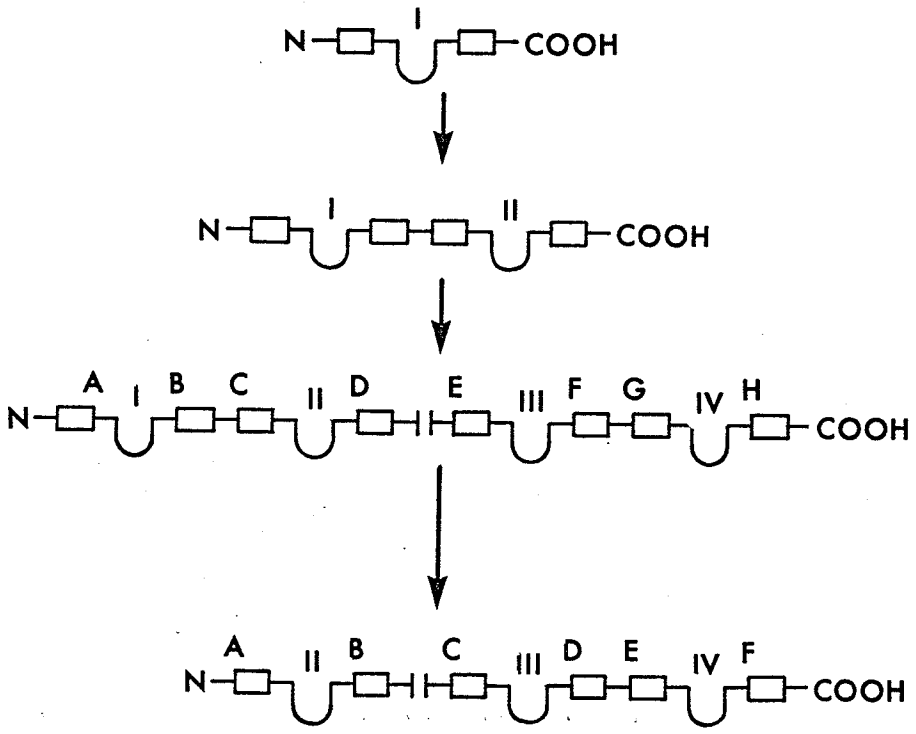
sheet conformation. The EF region is related to the CD region by an approximately two fold axis passing through the monomeric protein. The AB region lies over the EF and CD regions, does not bind Ca^{2+} but may function to cover and contribute to the hydrophobic core.

Reports in the literature of the existence of other proteins with homologous EF regions, such as troponin-C (Collins et al, 1973), alkali light chains of rabbit skeletal muscle (Weeds and McLachlan, 1974; Tufty and Kretsinger, 1975), DTNB light chains of rabbit skeletal muscle (Mutsuda et al, 1977) intestinal calcium binding protein (Hofman et al, 1977) and protein modulator (Vanaman et al, 1977) have suggested that the EF region represents the basic structural and homologous domain, repeated internally and shared by all members of the family of homologous proteins. Collins (1976a) has suggested that these homologous proteins were derived from an ancestral protein containing a single EF domain. This protein underwent two successive gene duplications and fusions to produce an ancestor containing four Ca^{2+}-binding sites. However, a comparison of the individual domains of the homologous proteins shows that regions two and four are more closely related to one another than they are to regions one and three. Vanaman et al, (1977)

and Kretsinger (1979) have suggested the existence of a primordial odd-even pair; the subsequent duplication would produce a pair of pairs, namely domains one and two and domains three and four. Gene fusion would complete the procedure. The model of the mechanism for the formation of the homologous proteins is presented in Scheme II (after Vanaman et al, (1977)).

In the case of parvalbumin it appears that domain one has been deleted after gene duplication, and domain two has lost the ability to bind Ca^{2+}. Parvalbumin contains two Ca^{2+}-binding sites with pK_d 6.5 (T = 25°C, pH 7.55, with 2 mM Mg^{2+}; Pechère, 1977) and in the absence of Mg^{2+} a pK_d of 8.4 was reported by Potter et al, (1977). A Hill coefficient of 1.05 suggests that at best only slight cooperativity in Ca^{2+}-binding may exist (Pechère, 1977),. Potter et al (1977)have reported that pK_d (Mg^{2+}) to be 4.0. Parvalbumin therefore contains two high affinity binding sites that bind Mg^{2+} competitively (Ca^{2+}-Mg^{2+} sites).

Skeletal muscle appears to be a rich source of parvalbumins. Amounts ranging from 11.5 g/Kg. (hake; Pechère et al, 1975) to 0.8 g/Kg (rabbit; Blum et al, 1977) have been reported. In addition to white skeletal muscle, values of less than 0.1 mg/Kg have been reported in carp brain (Gosselin-Rey et al, 1977), chicken red



SCHEME II

HYPOTHETICAL ANCESTRY AND STRUCTURAL FEATURES OF THE PROTEIN MODULATOR, TROPONIN-C AND PARVALBUMIN.

Kretsinger (1972) and Kretsinger and Nockolds (1973) first reported the internal sequence repeats of parvalbumin suggested gene triplication of a primitive Ca^{2+}-binding domain referred to as an "EF hand". Analysis of the sequence data of troponin-C, protein modulator ICBP, and myosin light chains have also demonstrated the existence of homologous internal repeat sequences of the EF region. Several investigators (Collins, 1976a; Vanaman et al, 1977; Kretsinger, 1979) have suggested that these homologous proteins are related to a common ancestral protein which arose from successive gene duplications of a 33 residue "EF" ancestor to form a protein of four "EF" regions. Analysis of the homology of the internal repeat sequences has suggested that the protein modulator is most closely related to the ancestral protein.

back muscle, breast, heart, stomach, brain, pancreas, small intestine and kidney but not spleen, liver, or blood (Heizmann et al, 1977). Baron et al, (1975) reported less than 0.2 mg/Kg of parvalbumin in rabbit uterus, bladder, small intestine, spleen, kidney, ovary, adipose tissue, lung, liver, and erythrocytes. Parvalbumin has also been found in the white leg muscle of the invertebrate Limulus (Anderson et al, 1978). Lowe et al, (1978) found by immunochemical techniques that in the mouse, parvalbumin is present only in white muscle. The results suggest that the majority of parvalbumin within an organism is located in the white muscle, but not restricted to this tissue.

The physiological function of parvalbumin is at present unknown. Pechère has suggested that parvalbumin may play a role in the relaxation cycle of white muscle as compensation for the kinetic insufficiencies of Ca^{2+} recaptured by the sarcoplasmic reticulum (Ebashi, 1976). Potter et al, (1977) suggested that parvalbumin may play a role in the activation of phosphodiesterase. The amount of parvalbumin necessary for half-maximal stimulation of phosphodiesterase (11 µg) was about 1400 x the amount of protein modulator required for similar activation. The fact that bovine serum albumin, also an acidic protein had no effect on the activity of phosphodiesterase at

concentrations up to 200 µg (per assay) suggested that the stimulatory nature of parvalbumin was not a non-specific ionic or acidic effect. The Ca^{2+} concentration required for half-maximal activation of phosphodiesterase by parvalbumin was determined to be 0.14 µM as compared to values of 1.2 µM for protein modulator activation, therefore suggesting that the activation of phosphodiesterase could not be due to a small contamination by protein modulator. Furthermore the Ca^{2+} concentration dependence of both Ca^{2+} binding and phosphodiesterase activation by parvalbumin were very similar, half-maximal stimulation of phosphodiesterase occurred with the binding of one mol Ca^{2+} (Potter et al, 1977).

2. Troponin-C

Comparative studies have shown that muscles in all animals are regulated by changes in Ca^{2+} concentration. Two distinctly different regulatory systems occur in different animals, one acting by means of the thin filaments (Ebashi, 1963) and the other by the thick filaments (Kendrick-Jones et al, 1970).

The term "thin filament control" refers to the fact that additional proteins required for the control mechanism -tropomyosin and troponin- are located on the thin filaments. Potter and Gergely (1974) have proposed a

mechanism for thin filament regulation of muscle contraction. In the presence of low levels of cytosolic Ca^{2+} ($10^{-7}M$) the troponin-tropomyosin complex blocks the interaction of actin and myosin. After stimulation the Ca^{2+} concentration increases ($10^{-5}M$), and the Ca^{2+}-binding subunit of troponin, troponin-C, binds Ca^{2+}, undergoes a conformational change ultimately resulting in the movement of tropomyosin into the long-pitch groove of actin, and allowing interaction of actin and myosin, thus, resulting in muscle contraction. A study of the properties of troponin-C is therefore fundamental in the understanding of the molecular mechanisms of the regulation of muscle contraction by Ca^{2+}.

The complete amino acid sequence of TN-C from rabbit (Collins et al, 1977) and chicken (Wilkinson, 1976) skeletal muscle and from cardiac muscle (van Eerd and Takahashi, 1976) has been determined. Several investigators (Collins et al, 1973; Weeds and MacLachlan, 1974; Tufty and Kretsinger, 1975) have noted the homology of TN-C to parvalbumin. Collins et al, (1974) first reported that the amino acid sequence of muscle TN-C could be divided into four homologous domains each of which contained a potential Ca^{2+}-binding site. Kretsinger and Barry (1975) have predicted a three dimensional model for TN-C, consisting of

two pairs of EF hands, each pair as found in parvalbumin. Since sequence analysis has suggested that parvalbumin evolved from a TN-C like ancestor with the C-terminal portion conserved, it is assumed that the two Ca^{2+} sites (CD and EF, scheme II) of parvalbumin correspond to sites III and IV of TN-C. Since the two parvalbumin sites are (Ca^{2+}-Mg^{2+}) sites this would mean that sites III and IV in skeletal muscle TN-C would also be (Ca^{2+}-Mg^{2+}) sites. This has been confirmed by Potter et al, (1976) and Leavis et al, (1977), using proteolytic fragmentation studies.

That skeletal muscle TN-C has two high affinity sites $pK_d(Ca^{2+})$ 7.3 which also bind Mg^{2+} pK_d 3.7 (Ca^{2+}-Mg^{2+} sites) and two low affinity sites $pK_d(Ca^{2+})$ 5.3, which do not bind Mg^{2+} (Ca^{2+} sites) was reported by Potter and Gergely (1975). In the presence of 2 mM Mg^{2+} the affinity of the (Ca^{2+}-Mg^{2+}) sites are lowered to $pK_d(Ca^{2+})$ of 6.3. Cardiac TN-C has two high affinity (Ca^{2+}-Mg^{2+}) sites (scheme II: III & IV) and one lower affinity site pK_d 4.3 in loop II (Potter et al, 1977). Loop I does not bind Ca^{2+}. The scatchard plot of the binding of Ca^{2+} by cardiac TN-C demonstrates positive cooperativity (Potter et al, 1977).

Studies of the myofibrillar ATPase activity as a function of Ca^{2+} concentration, at several Mg^{2+} concentrations have been examined by Potter and Gergely (1975)

who have concluded that since there is no difference in the Ca^{2+} dependence of myofibrillar ATPase at several Mg^{2+} concentrations, only the Ca^{2+} specific sites are involved in regulation of the myofibrillar ATPase. Using dansylaziridine (DANZ) labelled skeletal muscle TN-C as a probe of conformational changes due to Ca^{2+} binding by the Ca^{2+} specific sites, Potter et al, (1977) showed that the fluorescence of $TN\text{-}C_{DANZ}$ was enhanced approximately two fold when Ca^{2+} bound to the Ca^{2+}-specific sites, and this was accompanied by a 10 nm blue shift. The Ca^{2+} concentration required for half-maximal fluorescence change was 40 μM and was unaffected by the presence or absence of Mg^{2+}. Circular dichroism data have demonstrated that the Ca^{2+} dependent changes in ellipticity of skeletal muscle TN-C are biphasic with $K_{Ca}2+$ of 0.27 μM and 33 μM, representing binding to the (Ca^{2+}-Mg^{2+}) and Ca^{2+}-specific sites, respectively. The total change in α helix is from 34% to 50% (Kawasaki and van Eerd, 1972; Potter et al, 1977) and 35% of this change in ellipticity occurs with the binding of Ca^{2+} to the Ca^{2+} specific sites.

Troponin is a complex of three proteins (Greaser and Gergely, 1973). The components of the troponin complex are: troponin I (21,000 daltons) which is believed to inhibit the actin-myosin interaction, troponin C (18,000

daltons)which binds Ca^{2+} and relieves the inhibition caused by TN-I, and troponin T (30,500 daltons) which attaches the troponin complex to tropomyosin. Proteolytic studies (Leavis et al, 1977; Weeks and Perry, 1978; Drabikowski et al, 1977b) have indicated that the binding site of TN-C with TN-I is located between residues 83 - 104, and the binding sites for TN-T is located between residues 120 - 159. In terms of scheme I these binding sites are located in the III - IV domain.

TN-C has also been implicated in the Ca^{2+} dependent activation of phosphodiesterase (Dedman et al, 1977a) although 600 fold more TN-C (5 µg) is required to produce similar activation by protein modulator. The Ca^{2+} concentration required for half-maximal activation of phosphodiesterase by TN-C was 1.9 µM compared to 1.2 µM for the rat testes protein modulator (Dedman et al, 1977b). Since native muscle troponin at 100 µg concentration had no effect on phosphodiesterase activation, the activation of phosphodiesterase by TN-C was not a result of a 1/600 contamination by protein modulator.

The similarity in structure between TN-C and protein modulator has generated confusion in the literature, and made attempts to distinguish between these two proteins very difficult. As a result of this confusion reports have appeared in the literature concerning the isolation of soluble TN-C like proteins isolated from the extracts of

many tissues including bovine adrenal medulla (Kuo and Coffee, 1976), chick embryo brain (Fine et al, 1975), smooth muscle (Head et al, 1977), platelets (McGowan et al, 1976) and several vertebrate brains (Vanaman et al, 1975). Drabikowski et al, (1977b) has analyzed the 100,000 x g supernatant and pellet of the following tissues for the presence of TN-C, skeletal muscle (rabbit), cardiac muscle (bovine), smooth muscle (rabbit uterus and chicken gizzard), adrenal medulla (bovine), brain (bovine) and platelets (bovine). The partially purified cytosol or particulate fraction was subjected to preparative urea gel electrophoresis and the band revealing a common property of both TN-C and protein modulator namely the change in relative mobility in alkaline urea gel depending on the presence or absence of Ca^{2+} was isolated. TN-C and protein modulator were distinguished by three criteria, the difference in mobility on 15% SDS polyacrylamide gels, the difference in mobility of the complex with TN-I in urea gel in the presence of Ca^{2+}, and the ability of low concentration of protein modulator to stimulate Ca^{2+}-activatable phosphodiesterase. In all tissues examined TN-C was found only in the 100,000 x g pellet of skeletal and cardiac muscle, therefore indicating that the TN-C like proteins of smooth muscle (Head et al, 1977), brain (Vanaman et al, 1975), adrenal medulla (Kuo and Coffee, 1976) and platelet

(McGowan et al, 1976) are in fact the protein modulator. This suggestion has been confirmed; the TN-C like proteins isolated from bovine brain have been demonstrated to be the protein modulator (Stevens et al, 1976; Watterson et al, 1976) and the TN-C like protein isolated from soluble extracts of adrenal medulla has also been identified as the protein modulator (Crimaldi et al, 1978). Troponin however, may not be limited in distribution to vertebrate tissues; Goldberg and Lehman (1978) have reported the presence of troponin like proteins in the muscles of the scallop.

3. Protein Modulator

The amino acid sequence of the protein modulator from bovine brain has been determined by Vanaman et al (1977). The sequence of the protein modulator can be divided into four homologous domains each of which contains a potential Ca^{2+} binding site. The level of internal homology is greatest between domain one and domain three and between domain two and domain four. This level of internal homology appears greater than that observed within TN-C or parvalbumin (Collins, 1976a, b), and suggests that the protein modulator is more closely related to the ancestral protein (scheme II) than TN - C (Vanaman et al, 1977; Barker et al, 1977).

Alignment of the amino acid sequences of bovine

cardiac and rabbit skeletal muscle TN-C with that of bovine brain protein modulator has demonstrated that the modulator is closely related in structure to the muscle TN-C. Using those residues defined by Dayhoff (1976) as functionally conservative, Vanaman (1977) has determined that the total number of identical plus functionally conservative residues shared by bovine brain modulator and either TN-C, is 114 out of 148 positions compared (77% homologous), as compared with 82% homology between skeletal and cardiac TN-C. Walsh et al (1977) and Drabikowski et al (1977a) have demonstrated that tryptic cleavages in the protein modulator both in the presence and absence of Ca^{2+} occur in positions homologous to tryptic cleavage positions in TN-C. Collectively, these results confirm the original suggestion of Wang et al (1975) and Stevens et al (1976) that the protein modulator and TN-C are homologous proteins.

The Ca^{2+} binding properties of protein modulator from different tissues have been studied by many laboratories and there is no clear agreement about the affinity of the Ca^{2+} binding sites. However the literature supports the original discovery of Teo and Wang (1973) that the protein modulator binds four Ca^{2+} in the presence of 3 mM Mg^{2+}. This would suggest that unlike TN-C the protein modulator has four Ca^{2+} specific sites. Dedman et al (1977a) have

demonstrated that the binding of Ca^{2+} to the Ca^{2+} specific sites can produce the conformational changes (change in α helix from 45% to 54%) before activation of phosphodiesterase. Klee (1977) has suggested that the bulk of the conformational changes in the porcine brain protein modulator occurs upon the binding of the first 2 mol of Ca^{2+}. Dedman et al (1977b) have suggested that the binding of only one mol of Ca^{2+} is sufficient to result in PDE activation. Since the literature has not resolved the question of the existence of one or two classes of Ca^{2+} specific sites, the possible role of the four Ca^{2+} binding domains in the Ca^{2+} induced conformational changes of protein modulator cannot be evaluated. Dedman et al (1977a) have demonstrated that the protein modulator was capable of substitution for TN-C in the troponin complex, and regulation of actin-activated myosin ATPase.

Taken collectively the results suggest that the structural and functional properties of TN-C and protein modulator have been highly conserved; according to sequence determinations (Vanaman et al, 1977) TN-C and protein modulator are 77% homologous and each can substitute for each other in their respective systems.

4. Intestinal Calcium Binding Protein

Two classes of vitamin D-dependent calcium binding

proteins have been demonstrated, a 9,7000 molecular weight class found in mammalian intestine, bovine kidney, and guinea pig kidney, and a 28,000 molecular weight class found in avian intestine kidney, shell gland, brain, as well as bovine brain, rat kidney and human kidney. Within the two classes the proteins are immunologically cross reactive, however there is no cross reaction between the two groups. The chick gut and smaller bovine gut protein have been shown to be acidic proteins, pI 4.2 and 4.7 respectively (Wasserman et al, 1977). Collectively these proteins have been referred to as intestinal calcium binding proteins (ICBP).

While the exact cellular location of ICBP has not been resolved, a partly particulate (5%) and partly soluble (95%) subcellular distribution has been reported for the chick gut protein (Feher and Wasserman, 1976). The detergent dependent release of the particulate ICBP has been demonstrated, and since sonication and hypoosmotic treatment of washed cellular debris released only a small portion of the bound protein it is likely that the particulate ICBP is not due to contamination by the soluble form.

The amino acid sequence of porcine ICBP (Hofmann et al, 1977) has established the existence of two EF regions. The evolution of the ICBP is unclear. Kretsinger (1979) has pointed out a slight and possibly not significant closer

relationship of the two domains of ICBP to the domain I and II of the four domain proteins, protein modulator and TN-c, than to the domains III and IV of these proteins. This suggests that ICBP may have evolved by a deletion of a pair of EF domains. Alternatively, ICBP may have diverged prior to the basic pair duplication event and evolved directly from the original odd-even pair. The 28,000 dalton class of ICBP may represent a four domain protein, however, sequence studies will be necessary to evaluate these ICBP.

Evidence for the involvement of ICBP in Ca^{2+} transport has come from correlative data in which the rate of absorption of Ca^{2+} was shown to be directly related to the concentration of ICBP under a wide variety of conditions. Wasserman (1977) has demonstrated a temporal relationship between ICBP synthesis and Ca^{2+} transport after Vitamin D (1,25 dihydroxycholecalciferol) is given to rachitic chicks. The appearance of ICBP, initially preceeded the enhancement of Ca^{2+} absorption, then a parallel increase in both parameters occurred. Embryonic chick intestine culture studies have demonstrated that inhibitors of protein synthesis (actinomycin, α-amanitin) inhibit both the vitamin D stimulated synthesis of ICBP and the vitamin D dependent Ca^{2+} uptake (Corradino, 1973). Corradino et al (1976)

demonstrated that accompanying the vitamin D induced appearance of ICBP in chick intestine cells is an increase in Ca^{2+} uptake. Alternatively Krawit and Stubbert (1972) have demonstrated hydrocortisone inhibited vitamin D mediated absorption of Ca^{2+} in the rat, without a corresponding effect on the concentration of ICBP.

The results suggest that ICBP may be responsible for the vitamin D induced increase in intestinal Ca^{2+} transport, but the concentration of the ICBP in intestinal tissue may not account for the total intestinal response to vitamin D.

5. Myosin Light Chains

In muscle cells there are two well-described mechanisms by which Ca^{2+} regulates actin-myosin interaction. First, in skeletal muscle and also cardiac muscle, Ca^{2+} initiates the contractile event by binding to the regulatory protein, TN-C (Greaser and Gergely, 1973) which results in removal of inhibition of the actin-myosin interaction imposed by the complex troponin-tropomyosin, in the absence of Ca^{2+} (thin filament regulation). A second type of regulatory mechanism, originally elucidated for the scallop myosin, has been described by Szent-Gyorgi et al (1973). In this system Ca^{2+} regulation of actin-myosin interaction is mediated by a particular light chain

of myosin (thick filament regulation). Both types of regulation have been demonstrated in certain worms and insects (Lehman and Szent-Gyorgi, 1975).

Myosin is composed of two heavy chains (200,000 daltons) and four light chains (15,000 - 25,000 daltons). The presence of two distinct classes of light chains was first demonstrated in vertebrate skeletal muscle (Weeds, 1969; Weeds and Lowey, 1971) by chemical methods. One class of light chain (2 mol, molecular weight 19,000 daltons) could be dissociated from myosin by reaction with 5,5' dithiobis (2-nitrobenzoic acid) (DTNB). The dissociation of light chains from myosin does not result in the loss in myosin ATPase activity. (Weeds and Lowey, 1971). This light chain was termed the DTNB light chain and was not required for ATPase activity. The second class of light chain required alkaline pH for dissociation from myosin which resulted in a loss of enzyme activity (Weeds and Lowey, 1971). This light chain was referred to as the alkali light chain. Rabbit fast-twitch muscle myosin contains two alkali light chains of 21,000 and 17,000 molecular weight. They are identical in amino acid sequence over their C-terminal 141 residues but differ significantly in their N-terminal residues (Frank and Wedds, 1974). Furthermore, the alkali light chains occur in unequal and non integral

ratios (Lowey and Risby, 1971) and the relative amounts change during development (Pinnoni-Muller et al, 1976) therefore suggesting the presence of myosin isozymes within a single homogeneous muscle type. All other muscles examined do not have the two forms of this type of light chain, but contain two mol of identical light chain.

The scallop myosin ATPase system has allowed classification of the myosin light chains into two functional categories. In the presence of Ca^{2+} the scallop myosin ATPase activity can only be activated by actin when a full complement of light chains is present. Removal of one mol of a possible two mol of the EDTA light chain (so called because this light chain is removed by EDTA) results in a loss of Ca^{2+} sensitivity and one Ca^{2+} binding site. The removal of this light chain is called desensitization. Ca^{2+} sensitivity can be restored by readdition of the scallop EDTA light chain or by substitution of the 20,000 dalton light chain of smooth muscle, the 19,000 dalton light chain of vertebrate slow-twitch muscle, and the 19,000 dalton (DTNB) light chain of vertebrate fast-twitch muscle in place of the scallop (EDTA) light chain. In contrast the remaining class of light chain (e.g. the alkali light chains of vertebrate fast-twitch muscle) were ineffective in the restoration of Ca^{2+} sensitivity to scallop myosin (Kendrick-Jones et al, 1976).

The scallop myosin ATPase system has allowed a classification of all the myosin light chains according to function, into two classes, the regulatory light chains (RLC) and the essential light chains (ELC).

The regulatory light chains of all myosins restore Ca^{2+} sensitivity to the desensitized scallop actomyosin ATPase. They have been identified in all myosins. Comparisons of the amino acid compositions of these RLC (Kendrick-Jones et al, 1976) has suggested that these proteins are homologous. Furthermore, with the exception of the EDTA light chain, all RLC can be phosphorylated (Frearson and Perry, 1975; Perrie et al, 1973; Adelstein et al, 1976).

The essential light chains of myosin do not restore Ca^{2+} sensitivity to desensitized scallop actomyosin ATPase. Their name is derived from the fact that frequently the removal of these light chains results in loss of enzyme activity. Grouped in this category are the alkali light chains of fast-twitch myosin, the guanidine-HCl light chains of scallop myosin, the 17,000 dalton light chain of smooth muscle, and the 21,000 dalton light chains of cardiac and slow-twitch muscle. Recent work by Wagner and Weeds (1977) and Winstanley et al, (1977) has suggested that the ELC's do not have a significant effect on myosin ATPase activity in the absence of actin but may effect the actin activated myosin

ATPase activity and also may be involved in actin binding.

The amino acid sequence of the RLC of cardiac myosin (Léger and Elsinga, 1977), rabbit fast-twitch muscle, (Collins, 1976a), and scallop muscle (Kendrick-Jones and Jakes, 1977) as well as the ELC of fast-twitch muscle (Frank and Weeds, 1974) has been determined and compared with the sequences of parvalbumin, ICBP, TN-C, and protein modulator (Kretsinger, 1979). The results demonstrate that the myosin light chains are homologous both to each other and to the other members of the family of homologous proteins. The RLC and ELC both contain four EF domains. The pattern of internal sequence repeats is very weak, and the pattern of hydrophobic residues predicted to form the integral core has been conserved.

Of all the myosin light chains only the DTNB light chain can bind Ca^{2+}. This binding is weaker (K_d 10 μM) when Mg^{2+} is present (Werber et al, 1972). The Ca^{2+} binding site of the DTNB light chain therefore appears to be a (Ca^{2+}-Mg^{2+}) site. The desensitized scallop myosin preparation has been used to provide evidence that Ca^{2+} binding by the RLC may only occur when the light chain associates with myosin. Upon desensitization of scallop myosin both Ca^{2+} sensitivity and one Ca^{2+} binding site is lost. However when this preparation is resensitized with the

scallop EDTA light chain or by the RLC of vertebrate smooth muscle both Ca^{2+} sensitivity and Ca^{2+} binding are restored to the actin-activated myosin ATPase. This would suggest that the RLC of the scallop and smooth muscle demonstrate Ca^{2+} binding only when associated with myosin. Interestingly the Ca^{2+} binding exhibited by scallop myosin (K_d 1μM) is unaffected by Mg^{2+}, therefore indicating the possible presence of two Ca^{2+} specific sites on this myosin.

Sequence analysis of the site of phosphorylation of the RLC has suggested a serine residue located on the N-terminal side of domain I (Jakes et al, 1976). The EDTA light chain also contains an equivalent serine residue, but is not phosphorylated. Kendrick-Jones and Jakes (1977) have proposed that the N-terminal region of the EDTA light chain necessary for recognition by the kinase, is missing in the EDTA light chain (EDTA MW, 17,000; other RLC MW 19,000 - 20,000). Proteolytic studies have supported this postulate (Jakes et al, 1976). The RLC appear to be the only members of the family of homologous proteins which can be phosphorylated.

The evolution of the myosin light chains away from a direct Ca^{2+} regulatory role is suggested from analysis of the essential light chains. They do not bind Ca^{2+} or restore Ca^{2+} sensitivity to desensitized scallop myosin, nor are

they phosphorylated. However, sequence analysis (Frank and Weeds, 1974) have shown that the essential features of the family of the homologous proteins are retained, they possess EF regions and the distribution of hydrophobic residues in the EF regions have been conserved.

It is therefore reasonable to conclude that the RLC and the ELC of myosin have evolved from a common ancestral protein, as have parvalbumin, TN-C, and protein modulator. Interestingly, analysis of the homology of the internal repeat sequences has suggested that the protein modulator is probably the most closely related member of this family of homologous proteins to the ancestral protein (Barker et al, 1977; Kretsinger et al, 1979).

IV. PHYSIOLOGICAL SIGNIFICANCE OF PROTEIN MODULATOR REGULATION

1. Cyclic Nucleotide Phosphodiesterase

Cyclic nucleotide phosphodiesterase was first identified by Butcher and Sutherland (1962) in bovine heart, and represents the only enzymatic mechanism for the hydrolysis of c-AMP and c-GMP. That rat cerebral cortex contained multiple forms of cyclic nucleotide phosphodiesterase was first reported by Thompson and Appleman (1971). Two or more forms of the enzyme have been found in every tissue examined. Methods that have been used to characterize these multiple

forms include gel filtration (Thompson and Appleman, 1971; Kakiuchi et al, 1971), anion exchange chromatography (Russel et al, 1973; Schubart et al, 1974; Wells et al, 1975) polyacrylamide gel electrophoresis (Goren et al, 1971; Uzunov and Weiss, 1972; Campbell and Oliver, 1972) starch gel electrophoresis (Monn and Christiansen, 1971), sucrose density gradient centrifugation (Thompson et al, 1973) and isoelectric focussing (Pledger et al, 1974).

Russel et al (1973) have demonstrated that DEAE-cellulose chromatography of liver extract prepared by homogenization, sonication, and centrifugation, exhibits three discrete active fractions of phosphodiesterase activity referred to as D-I, D-II, and D-III according to their elution from the column by a salt gradient. This chromatographic analysis has been applied to a number of mammalian tissues including heart, kidney, mammary gland, lung, and brain (Appleman and Terasaki, 1975) and while the relative amounts of the different forms of phosphodiesterase activities vary from tissue to tissue the basic pattern of three fractions of cyclic nucleotide phosphodiesterase activity is conserved. Other forms of phosphodiesterase activity in addition to the three described above may exist in mammalian tissues. For example, six fractions of phosphodiesterase activity have been isolated from sonicated cerebellar preparations by

polyacrylamide gel electrophoresis (Uzunov and Weiss, 1972) and by isoelectric focussing (Pledger et al, 1974). Hidaka et al, 1978 has reported the separation of five fractions of PDE activity by DEAE-cellulose chromatography. While these fractions could be separate enzymes it is also possible they could represent forms that arise from proteolysis or from aggregation of one or more of the original forms present.

Of the three forms of phosphodiesterase activity originally reported by Russel et al (1973), D-III appears to have a low Km (c-AMP), particulate enzyme. The activity of D-III is characterized by relative selectively toward c-AMP as substrate and in many studies by concave-downward Linweaver-Burk plots (Appleman et al, 1973). Furthermore, the activity of this enzyme may change in rat liver in response to insulin and glucagon (Loten et al, 1978). While the D-III enzyme of rat liver is particulate, forms D-I and D-II appear to be soluble. D-II has been identified as possessing approximately equal activity toward c-AMP and c-GMP. When c-GMP is used as substrate the Linewearer-Burk plot shows a pronounced upward curvature suggesting the presence of homotropic cooperativity in the enzyme with respect to this substrate, however no significant homotropic interactions were reported when c-AMP was the substrate. This enzyme

has been characterized in rat liver (Appleman and Terasaki, 1975) and bovine heart (Ho et al, 1976).

The separation of phosphodiesterase into a Ca^{2+} sensitive and Ca^{2+} insensitive form was initially reported by Kakiuchi et al (1971), in rat brain. Similar results have been obtained for bovine heart (Goren and Rosen, 1972; Hrapchak and Rasmussen, 1972; Wang et al, 1972) and rat liver and heart (Appleman and Terasaki, 1975). Using the nomenclature of Russel et al (1973), kinetic analysis has confirmed that the Ca^{2+} sensitive enzyme corresponds to D-I and the Ca^{2+} insensitive form to D-II. The activity of D-I is stimulated by the protein modulator and the activation is completely reversed by EGTA (Kakiuchi et al, 1973; Teo and Wang, 1973). All reports to date are consistant with the idea that a single protein modulator sensitive enzyme will catalyse the hydrolysis of both c-AMP and c-GMP. Reports that the hydrolysis of c-AMP is competitively inhibited by c-GMP and vice versa, with K_i values that are similar to the respective Km values strongly suggest that a single catalytic site is involved in the hydrolysis of both substrates (Wells et al, 1975b; Brostrom and Wolff, 1976; Ho et al, 1976). Ho et al (1977) have reported a specific activity of 120 µmol/min/mg for a partially pure (80%) phosphodiesterase preparation. Ca^{2+}-activable phosphodiesterase has been purified from

bovine brain by two groups of investigators (Wallace et al, 1978; Klee et al, 1978).

In all tissues examined thusfar, it has been shown that the Ca^{2+}-activated phosphodiesterase has a higher affinity for c-GMP than c-AMP. These tissues include rat brain (Kakiuchi et al, 1973), porcine brain (Brostrom and Wolff, 1976), bovine heart (Ho et al, 1975), rat heart (Appleman and Terasaki, 1975), porcine coronary arteries (Wells et al, 1975b), and human aorta (Hidaka et al, 1978). These observations have led to the suggestion that the Ca^{2+}-activated enzyme is primarily a c-GMP enzyme in vivo.

The kinetic mechanism of phosphodiesterase activation is at present unclear. Ho et al, (1976) have demonstrated that the activation of bovine heart phosphodiesterase by the protein modulator results in a five fold increase in the Vmax and a decrease of about 90% in the Km (from 1.5 mM to 0.2 mM) when c-AMP is used as substrate. Similar results have been reported by Klee et al, (1978). When c-GMP is used as substrate, a decrease in Km of about fifty fold (from 0.26 mM to 9 μM) and no change in Vmax (remains at about 30% of the rate of hydrolysis of c-AMP by the modulator stimulated enzyme) was reported by Ho et al, (1976). Brostrom and Wolff (1976) have reported that protein modulator activation of PDE results only in a decrease in Km when both substrates are used.

In contrast, Wickson et al (1975)have reported a change in Vmax without any change in Km when c-GMP is used as substrate. The reason for these conflicting observations is not at all apparent but may reflect tissue differences, different states of purity, or different assay conditions.

The Ca^{2+}-stimulated phosphodiesterase can also be activated by proteolysis (Cheung, 1971) and by certain lipids and phospholipids, particularly phosphatidyl inositol and lysolecithin (Wolff and Brostrom, 1976), but this stimulation is independent of Ca^{2+}. Hidaka et al, 1978 have reported stimulation of human aorta PDE by behenic acid (C_{22}). Imidazole and ammonium sulfate (Ho et al, 1976) have have been reported to specifically increase the maximal activation of phosphodiesterase by the protein modulator. Mg^{2+} has been demonstrated to inhibit PDE by competing with Ca^{2+} for the protein modulator (Ho et al, 1976). Levin and Weiss (1977) have demonstrated that the inhibition of PDE by the antipsychotic drug trifluoperazine results from direct binding of trifluoperazine to the protein modulator. The effects of other phosphodiesterase inhibitors e.g. theophylline, and papaverine have been suggested to act by competition with the substrate (Weiss, 1975), and to vary in effectiveness from tissue to tissue according to the pattern of phosphodiesterase activities found in these tissues.

2. Adenylate Cyclase

Adenylate cyclase derived from a variety of tissues has been shown to be strongly inhibited by low concentrations of Ca^{2+} (Birnbaumer, 1973). Brain tissue (Bradham et al, 1970) and one glioma cell line (Brostrom et al, 1976) however, possess adenylate cyclase activity which exhibits a biphasic response to Ca^{2+}; low Ca^{2+} concentrations activate and higher concentrations inhibit the activity. Using ECTEOLA-cellulose chromatography to partially purify detergent-dispersed extracts of brain tissue, several investigators have demonstrated the existence of PM activated adenylate cyclase in porcine cerebral cortex (Brostrom et al, 1975) and rat or bovine brain (Cheung et al, 1975b). The adenylate cyclase activity of a rat cerebral cortex preparation has been shown to be composed of two contributing components, only one of which requires the protein modulator for activity (Brostrom et al, 1977). The protein modulator component represented 80% of the basal activity of the cortex homogenate and comprised about half of the overall activity when each component was fully activated by NaF. Pretreatment of this preparation with Ca^{2+} and modulator for 1 hr at 37^{o} resulted in the selective inactivation of the protein modulator independent adenylate cyclase, and therefore allowed a study of the protein modulator dependent adenylate cyclase activity. The

PM-dependent enzyme was shown to be activated 10 to 30 fold by PM, to respond biphasically to free Ca^{2+} concentrations (in the presence of PM), to be stimulated by NaF only in the presence of Ca and PM, NaF stimulation being readily reversed by EGTA, to be inhibited by high ratios of Mg^{2+} to Ca^{2+} and to be inhibited by chloropromazine. In contrast, the PM-independent adenylate cyclase was inhibited with increasing free Ca^{2+} concentration, had elevated activity at high ratios of Mg^{2+} to Ca^{2+}, was not effected by chloropromazine, and was irreversibly activated by NaF.

The activation of adenylate cyclase by protein modulator is very similar to the activation of phosphodiesterase. The activation of adenylate cyclase by PM is Ca^{2+} dependent and reversible by EGTA (Brostrom et al, 1975). Activation depends on the simultaneous presence of Ca^{2+} and PM (Brostrom et al, 1975; Cheung et al, 1975), and the formation of the PM-adenylate cyclase complex is dependent on Ca^{2+} (Lynch et al, 1976). Lynch et al (1976b) have reported that the protein modulator increased the Vmax several fold but did not effect the Km for ATP. Stimulation required Ca^{2+}, with half-maximal effect at 15 μM. That the PM and Ca^{2+} conferred thermal stability to adenylate cyclase indicates that the protein modulator probably induced a conformational change in the enzyme, an increase in thermal stability of phosphodiesterase

preparations due to the protein modulator and Ca^{2+} has also been reported (Ho et al, 1976).

3. Cyclic Nucleotide Regulation

The fact that the protein modulator stimulates the activities of both adenylate cyclase (catalyzing the formation of c-AMP and cyclic nucleotide phosphodiesterase (catalyzing cyclic nucleotide degradation) appears paradoxical. A further complication is added by the observation of protein modulator sensitive and insensitive forms of phosphodiesterase and adenylate cyclase activity. While protein modulator stimulated phosphodiesterase has been reported in most mammalian tissues examined, protein modulator stimulated adenylate cyclase has only been reported in mammalian brain. For these reasons, the exact physiological mechanism of the regulation of cyclic nucleotide metabolism by the protein modulator remains speculative.

One of the earlier attempts to postulate a possible physiological function for protein modulator in the regulation of cyclic nucleotide metabolism was by Kakiuchi et al (1973). They proposed that based on kinetic evidence the modulator dependent phosphodiesterase is actually a c-GMP rather than a c-AMP hydrolyzing enzyme. Therefore the Ca^{2+} influx in response to a stimulus was suggested to result in the formation of an active Ca^{2+} protein modulator complex

resulting in the simultaneous stimulation of adenylate cyclase and phosphodiesterase, causing an increase in intracellular c-AMP and a decrease in c-GMP.

Another theory, proposed by Lynch et al (1976) suggests that the sequential activation of adenylate cyclase and phosphodiesterase causes a transient elevation of c-AMP in many tissues. Stimuli which result in an influx of Ca^{2+} through the plasma membrane, or release of membrane bound Ca^{2+} would activate the membrane bound adenylate cyclase resulting in an increase of intracellular c-AMP. The Ca^{2+} would then activate the soluble phosphodiesterase therefore decreasing the c-AMP to basal concentrations. Bartfai (1978) has proposed that protein modulator regulation of phosphodiesterase and adenylate cyclase may be of importance in the regulation of c-AMP and perhaps c-GMP in postsynaptic neurons. Uzunov et al (1975) have reported that stimulation of the nicotinic receptors of adrenal medulla by carbamylcholine resulted in elevation of the content of c-AMP and protein modulator, the increase in c-AMP preceeded the increase in protein modulator. Furthermore, when the increase of the protein modulator reached peak values, the c-AMP content of the medulla was declining. Subsequently Uzunov et al (1976) demonstrated that the protein modulator released from the cytosol interacted with the high K_m phosphodiesterase in the presence of Ca^{2+}, and lowered its K_m

thereby facilitating the hydrolysis of c-AMP. Gnegy et al (1976) demonstrated that protein modulator released from the membrane fraction of a brain or adrenal medulla homogenate could be stimulated by in vitro incubation with purified c-AMP dependent protein kinase. The c-AMP dependent protein kinase did not phosphorylate the protein modulator, therefore suggesting phosphorylation of the membrane preparation. Subsequently Gnegy et al (1977) reported that the release of protein modulator was mainly from membrane fractions enriched in synaptic membranes, and occurred due to the phosphorylation of membrane protein by the c-AMP dependent protein kinase. From this evidence Gnegy et al (1977) proposed that the protein modulator was directly involved in the transsynaptically elicited increase in c-AMP in neuronal tissue. It was postulated that when the concentration of c-AMP reaches a certain level it activates the c-AMP dependent protein kinase which phosphorylates a membrane protein that binds protein modulator, resulting in release of protein modulator, stimulation of phosphodiesterase, and a decrease to resting levels of c-AMP.

4. $(Ca^{2+}-Mg^{2+})$ ATPase

A soluble factor capable of the stimulation of the $(Ca^{2+}-Mg^{2+})$ ATPase of the human erythrocyte membrane was originally reported by Bond and Clough (1973). Luthra

et al (1976) partially purified this activator and reported that it was acidic, acid and heat stable, and had a molecular weight below 50,000. The interaction of the red blood cell activator with the membrane resulted in the stimulation of the (Ca^{2+}-Mg^{2+}) ATPase, and this interaction between the activator and the membrane was demonstrated to be dependent on the presence of Ca^{2+} (Farrance, 1976). Noting the similarity of the red blood cell activator with the Ca^{2+} binding proteins, parvalbumin troponin-C and protein modulator, Gopinath and Vincenzi (1977) tested these proteins as potential activators of the (Ca^{2+}-Mg^{2+}) ATPase. While parvalbumin and troponin-C produced a modest increase in activity at high concentrations, the biological activity of the red blood membrane activator and the bovine brain protein modulator (1.5 µg/ml) were indistinguishable, both proteins produced about a four fold activation, and increased the Vmax and apparent affinity of (Ca^{2+}-Mg^{2+}) ATPase for Ca^{2+}. Based on these observations Gopinath and Vincenzi suggested that the protein modulator and the red blood cell activator might be the same protein.

Independently, Janett and Penniston, (1977) also reported activation of the human erythrocyte (Ca^{2+}-Mg^{2+}) ATPase by a soluble endogenous activator. This activator was purified 475-fold by these investigators who reported

that the ATPase activator and the protein modulator were very similar, both stimulated (Ca^{2+}-Mg^{2+}) ATPase and phosphodiesterase and they co-electrophorised on SDS polyacrylamide gel.

Recently Jarrett and Penniston (1978) have reported purification of the human erythrocyte activator to homogeneity. A comparison of the protein modulator and the human erythrocyte activator was undertaken; the amino acid compositions of the proteins were nearly indistinguishable and both proteins contained one residue trimethyllysine. Furthermore, both proteins were indistinguishable as measured by their ability to activate the (Ca^{2+}-Mg^{2+}) ATPase (values for 50% of maximal activation of the (Ca^{2+}-Mg^{2+}) ATPase varied from 36 to 76 ng/ml, depending on the history of the ghosts).

The erythrocyte membrane (Ca^{2+}-Mg^{2+}) ATPase activity is thought to be an expression of the Ca^{2+} active transport system in whole cells (Schatzmann and Vincenzi, (1969) which functions to maintain low intracellular Ca^{2+} concentrations. Because of the Ca^{2+} dependent and reversible binding to the erythrocyte membrane, and specificity of the activation of the (Ca^{2+}-Mg^{2+}) ATPase it has been postulated that the protein modulator functions as a regulator of the plasma membrane Ca^{2+} pump (Farrance et al,

1977). MacIntyre and Green (1977) observed stimulation of Ca^{2+} transport of an inside-out vesicle preparation of human erythrocyte by lysed erythrocyte supernatant. Hinds et al (1978) have tested this hypothesis with inside-out membrane vesicles prepared from human red blood cells. The active transport of Ca^{2+} was increased by addition of the human erythrocyte activator or the bovine brain protein modulator. When Lanthanum, an inhibitor of active Ca^{2+} transport (Quist and Roufogalis, 1975) was added to the system in the presence of protein modulator, the uptake of Ca^{2+} was inhibited. Addition of the calcium ionophore A23187 caused a rapid effux of Ca^{2+} from the loaded vesicles. The results suggest that the activity of the human erythrocyte plasma membrane Ca^{2+} pump may be regulated by the protein modulator.

5. Modulator Binding Proteins

Recently two bovine brain proteins which exhibit specific inhibitory activity against the protein modulator activated cyclic nucleotide phosphodiesterase have been reported, one of the proteins is heat labile and is referred to as the modulator binding protein (MBP) (Wang and Desai, 1976; Klee and Krinks, 1978), the other protein is heat stable and referred to as the heat stable inhibitor protein (HSIP) (Sharma et al, 1978a).

A. Modulator Binding Protein

The MBP specifically counteracts the activation of phosphodiesterase by the protein modulator, and shows no inhibitory activity, against the basal activity of the Ca^{2+}-activatable enzyme, against the trypsin activated Ca^{2+}-activatable enzyme, or against the Ca^{2+}-insensitive enzyme (Wang and Desai, 1977). Two experimental approaches have suggested that the MBP interacts with the protein modulator in the presence of Ca^{2+} (Wang and Desai, 1977). First, kinetic studies of the mutual effects of protein modulator and the MBP in the phosphodiesterase reaction are characteristic of a competitive interaction; the inhibition of PDE by MBP, is reversed by the addition of excess PM.

Second, gel filtration analysis (Sephadex G-200) has revealed that in the presence of Ca^{2+} the protein modulator and MBP can associate to form a complex; no interaction between Ca^{2+}-activatable phosphodiesterase and the MBP in the presence of EGTA was observed. These results have suggested that the MBP is similar to other protein modulator regulated enzymes, such as Ca^{2+}-activatable phosphodiesterase and adenylate cyclase in forming a Ca^{2+} dependent complex with the protein modulator. While MBP might possess catalytic activity, at present no catalytic activity has been identified for this protein. Wang and Desai (1977)

have tested MBP for various enzyme activities, including ATPase, GTPase, 5'nucleotidase and adenylate cyclase. Results were negative. It therefore remains unsettled whether MBP represents a protein modulator regulated enzyme or protein with nonenzymatic activity such as specific binding or transporting activity. It also cannot be ruled out that MBP might represent *in vivo* regulation of PM.

The modulator binding protein of bovine brain has been purified, to near homogeneity by Sharma *et al* (1979b), by a procedure based on the Ca^{2+}-dependent association of this protein with the protein modulator. The MBP is shown to be a globular protein (stokes radius 40.5^{o}A) of molecular weight 85,000. It has been shown to contain two distinct subunits of molecular weight 60,000 (α) and 14,500 (β) daltons respectively. A mass ratio of subunits α/β of 2.3 has been interpreted to suggest the subunit structure of MBP is α/β. Sharma *et al* (1979b) have separated the subunits of MBP by gel filtration on Sephadex G-100 in the presence of 6 M urea. Characterization of the separated subunits has demonstrated that only the α subunit can interact with PM. Furthermore the interaction of a subunit of MBP and PM is unaffected by β subunit. The results suggest that subunit α of the MBP is responsible for association of the MBP and the protein modulator. The function of the β subunit is at present unresolved.

B. Heat Stable Inhibitor Protein

A heat stable inhibitory protein of Ca^{2+} activatable phosphodiesterase has been partially purified from bovine brain by Sharma et al, (1978a). The HSIP has a molecular weight of 70,000 daltons as determined by gel filtration on G-100 Sephadex, and therefore appears to be different from the heat stable phosphodiesterase inhibitor of bovine retina (Dumler and Etinof, 1976) (MW 40,000).

The mechanism of action of HSIP is similar to that of the MBP. Based on the competitive interaction between HSIP and modulator protein in the phosphodiesterase reaction, and the Ca^{2+} dependent association of HSIP and protein modulator on gel filtration columns (G-100 Sephadex), Sharma et al (1978b) has suggested that the HSIP inhibits phosphodiesterase by specifically interacting with the protein modulator.

The HSIP has been purified to electrophoretic homogeneity by Sharma et al (1978b). The HSIP had a molecular weight of 68,000 on SDS polyacrylamide gels therefore suggesting the HSIP is monomeric. The protein has no inhibitory activity toward the c-AMP dependent protein kinase or protein phosphatase. Furthermore, examination of HSIP for various enzyme activities includint ATPase, GTPase, c-AMP phosphodiesterase, c-GMP phosphodiesterase, 5'nucleotidase and protein kinase has proved to be negative. It

therefore appears that the physiological function of both MBP and HSIP is unknown. These proteins could represent *in vivo* regulators of protein modulator, protein modulator regulated enzymes of unknown catalytic function or protein modulator regulated proteins (non enzymatic activity).

6. Stimulus-Secretion Coupling

Considerable evidence suggests that Ca^{2+} plays a number of important roles in the functioning of the nervous system (Rubin, 1970; Baker, 1972). Electrical depolarization of nerve terminals and of other secreting structures has been shown to cause an influx of extracellular calcium through specific calcium channels (Baker *et al*, 1971) and this calcium influx appears to mediate the stimulus-coupled release of neurotransmitters and peptide hormones (Katz and Miledi, 1967; Douglas, 1968). There is also evidence that calcium may regulate the synthesis of catecholamines in presynaptic nerve terminals (Patrick and Barchas, 1974).

A possible mechanism for the Ca^{2+} regulated release of neurotransmitter from presynaptic nerve terminals has been suggested by several investigators. DeLorenzo (1976) has reported that in preparations of ruptured rat brain synaptosomes two proteins of molecular weight 60,000 and 50,000 daltons are phosphorylated by a mechanism which is stimulated by calcium and inhibited by diphenylhydantoin

(DPH). Krueger et al (1977) has similarly reported that agents known to increase Ca^{2+} transport across the plasma membranes of nerve terminals, stimulate the phosphorylation of two specific endogenous proteins in intact synaptosomes (80,000 and 86,000 daltons). Agents found to be stimulatory included veratridine, high K^{+} (60 mM), and the calcium ionophore A23187. The results were interpreted to suggest that conditions which cause an accumulation of Ca^{2+} by synaptosomes lead to a calcium-dependent increase in phosphorylation of specific endogenous proteins and that these phosphoproteins may be involved in the regulation of certain calcium-dependent nerve terminal functions such as neurotransmitter synthesis and release.

That the molecular mechanism mediating the effects of calcium on neurotransmitter release and synaptic vesicle function may be the action of calcium on the level of phosphorylation of specific synaptic vesicle-associated proteins was first suggested by DeLorenzo and Freedman (1977a, b). These investigators presented evidence to show that the Ca^{2+} specific phosphorylated proteins are present in the presynaptic nerve terminal. A direct correlation between Ca^{2+} specific synaptosomal neurotransmitter release and Ca^{2+} specific synaptosomal phosphorylation has been reported by DeLorenzo and Freedman (1978). Using highly purified synaptosomal preparation these investigators

have demonstrated that both Ca^{2+} and Mg^{2+} are necessary for synaptosomal phosphorylation, and norepinephrine release. Ca^{2+} or Mg^{2+} alone was ineffective. Ca^{2+} was also shown to cause the greatest increase in the levels of phosphorylation of synaptosomes that showed the greatest Ca^{2+} dependent norepinephrine release. DPH was demonstrated to inhibit both the Ca^{2+} dependent neurotransmitter release (Pincus and Lee, 1973) and Ca^{2+} dependent synaptosomal phosphorylation. Furthermore, it was shown that both the Ca^{2+}dependent phosphorylation and Ca^{2+} dependent neurotransmitter release were stimulated by a protein factor. The results are compatible with the hypothesis that the phosphorylation of specific synaptic vesicle proteins is the molecular mechanism mediating some of the effects of calcium on neurotransmitter release. De Lorenzo and Freedman (1978) have also reported similar results for the Ca^{2+} dependent release of dopamine, acetylcholine, and γ aminobutyric acid.

Schulman and Greengard (1978) have reported that the calcium-dependent phosphorylation of highly purified membrane fractions from rat cerebral cortex required an endogenous heat stable protein factor present in the synaptosomal cytoplasm. Calcium stimulated phosphorylation lost on purification of synaptic membranes could be effectively recovered by reconstitution with either the synaptosomal

cytoplasm or a purified preparation of protein modulator. The PM regulated kinase was localized in the synaptosomal membrane.

The results of De Lorenzo and Friedman (1978) and Schulman and Greengard (1978) have suggested that the Ca^{2+} dependent presynaptic release of neurotransmitter is dependent on the phosphorylation of specific presynaptic proteins by a particulate, protein modulator regulated protein kinase.

Ca^{2+} has also been shown to regulate the synthesis of catecholamines in presynaptic nerve terminals (Patrick and Barchas, 1974). Kuhn et al (1978) and Hamon et al (1978) have presented evidence that tryptophan hydroxylase in rat brainstem extracts is activated by a Ca^{2+} stimulated protein kinase. Phosphorylation increased the catalytic activity of the enzyme 2.5 - fold and decreased the apparent Km for cofactor. Morgenroth et al (1975) have observed a reversible four fold increase in tyrosine hydroxylase activity in rat brain extracts with an apparent pK_d (Ca^{2+}) of 5.9. However, only c-AMP dependent phosphorylation of tyrosine hydroxylase has been reported (Yamauchi and Fujisawa, 1978). The c-AMP dependent phosphorylation of tyrosine hydroxylase is compatible with the postulated stimulation of norepinephrine release by presynaptic β-adrenoreceptors (Pelayo et al, 1978).

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It is interesting to note that while the anticonvulsant drug diphenylhydantoin has been shown to inhibit Ca^{2+} dependent phosphorylation and Ca^{2+} dependent release of neurotransmitter from synaptosomes (De Lorenzo and Friedman, 1978) this drug has also been demonstrated to block several other Ca^{2+}-dependent release processes including the release of insulin from islet cells (Knopp et al, 1972), and of oxytocin (Mittler and Glick, 1972), and antidiuretic hormone release from the pituitary. It is within the realm of possibility that protein modulator regulated protein kinase phosphorylation represents a basic mechanism for the Ca^{2+} mediated process of stimulus-secretion coupling. It is however apparent that DPH appears to represent an agent capable of inhibition of many Ca^{2}— dependent secretory processes, at least one of which is regulated by protein modulator.

7. Stimulus-Contraction Coupling

Considerable evidence suggests that Ca^{2+} is the coupling agent between excitation and contraction in all forms of muscle (Bianchi, 1973). In different types of muscle or contractile systems the major source of calcium which initiates the contractile process differs. Recent developments in muscle biochemistry have suggested that the protein modulator may mediate the effects of Ca^{2+}, although

the role of the protein modulator appears to differ in different types of muscle.

A. Smooth Muscle

That myosin could be responsible for the regulation of actin-myosin interaction (thick filament regulation) by Ca^{2+} was first demonstrated in molluscam muscles (Kendrick-Jones et al, 1970) and from comparative studies (Lehman et al, 1972) was initially considered to be a property exhibited only by myosin from certain invertebrate species. More recently, studies of vertebrate smooth muscle have indicated that in this system, Ca^{2+} regulation is also a myosin-linked phenomena (Bremel, 1974). Sobieszek and Small (1976) have extended the observations of Bremel (1974). They have shown that smooth muscle myosin (chicken or turkey gizzard) binds Ca^{2+} and interacts with F-actin from either smooth or skeletal muscle to produce actomyosins that exhibit a Ca^{2+}-dependent actin-activated ATPase. Mixtures of smooth muscle thin filaments with skeletal muscle myosin were Ca^{2+} insensitive (ie. no thin filament regulation). Furthermore, the results of proteolytic digestion experiments suggested that the 20,000 dalton light chain (RLC) of the chicken or turkey gizzard myosin might be necessary for Ca^{2+} sensitivity. More direct evidence for the involvement of the RLC chain of smooth muscle in the Ca^{2+} regulatory process

has come from the demonstration (Sobieszek, 1977) that phosphorylation of this light chain is triggered by the same Ca^{2+} concentration required to activate the ATPase activity of actomyosin. The degree of phosphorylation of the myofibrils was proportional to their measured Ca^{2+} sensitivity. The phosphorylation process was very rapid and essentially completed before the rise in ATPase activity. While the enzyme responsible for the Ca^{2+} dependent phosphorylation was identified as a specific myosin light chain kinase, attempts at purification of the enzyme resulted in a loss of enzyme activity. Ikebe et al (1977) also reported Ca^{2+} dependent phosphorylation of the RLC of myosin, and in addition, dephosphorylation of myosin by a crude preparation of myosin light chain phosphatase.

The myosin light chain kinase has been purified by Dabrowska et al, (1977). They have reported that the kinase is composed of two proteins of molecular weights 105,000 and 17,000 daltons, none of which alone possessed any activity. Dabrowska et al (1978) have identified the 17,000 dalton component as the protein modulator. The results have therefore suggested that the protein modulator mediates the Ca^{2+} dependent phosphorylation of the RLC of smooth muscle. The results of Sobieszek (1977) and Dabrowska et al, (1978) suggest the mechanism of regulation of actin-myosin interaction in smooth muscle is based on the phosphorylation

and dephosphorylation of myosin. When a muscle cell is stimulated the Ca^{2+} concentration rises and forms an active Ca^{2+}-PM complex which serves to activate the myosin light chain kinase resulting in phosphorylation of the 20,000 dalton RLC of myosin, and activation of the actin-activated myosin ATPase. When the Ca^{2+} concentration is returned to resting levels, the myosin light chain kinase is inactive (the PM stimulation is removed) and the activity of an as yet uncharacterized phosphatase dephosphorylates myosin, resulting in loss of the actin activated myosin ATPase and muscle relaxation.

It is interesting to note that in scallop myosin, the first system to demonstrate myosin regulation of muscle contraction, phosphorylation of the RLC is not thought to occur. Instead it has been proposed that the binding of Ca^{2+} by the RLC of scallop myosin (in association with myosin) is the required event to allow actin and myosin interaction. The scallop myosin therefore represents a system in which Ca^{2+} exerts a regulatory role by direct interaction with the Ca^{2+} binding component of myosin, namely the regulatory light chain.

Adelstein et al,1977 have pointed out that Ca^{2+} dependent phosphorylation of the RLC of guinea pig vas deferens myosin results in increased actin-activated

myosin ATPase activity, while dephosphorylation decreases this activity. However, unlike vertebrate smooth muscle the actin-activated myosin ATPase activity of phosphorylated vas deferens myosin was inhibited by EGTA in the absence of kinase and phosphatase. The results suggest that the vas deferens myosin may be regulated by both Ca^{2+} dependent phosphorylation of the RLC (as is vertebrate smooth muscle) and direct interactions of Ca^{2+} with the RLC of myosin (as is molluscan myosin).

B. Skeletal Muscle

The original observation that myosin from rabbit skeletal muscle was a phosphoprotein (Perrie et al, 1972) sparked interest in the possible regulatory role of protein phosphorylation in myofibrils. Subsequently, Perrie et al (1973) identified that the RLC (the DTNB light chain) of myosin was phosphorylated at a single serine residue by the action of a kinase present in the sarcoplasm. That this 18,000 dalton RLC was phosphorylated by a specific Ca^{2+}-dependent light chain kinase was reported by Pires et al (1974). The myosin light chain kinase was purified to electrophoretic homogeneity by Pires and Perry (1977), who suggested that the enzyme was monomeric with a molecular weight of 77,000 daltons. In contrast, Yazawa and Yagi (1977) reported that rabbit skeletal muscle

myosin light chain kinase was composed of two protein components of molecular weight 100,000 and 20,000 daltons. The 20,000 dalton component was identified as a Ca^{2+}-binding protein. Independently Yagi et al (1978) and Waisman et al (1978) identified this Ca^{2+}-binding protein as the protein modulator. Therefore, in the presence of modulator protein, the activity of the myosin light chain kinase, and hence light chain phosphorylation is reversibly controlled by Ca^{2+}.

The phosphorylation of the RLC of rabbit skeletal muscle myosin in vivo has been investigated by Stull and High, (1977). They have reported that the light chains of myosin contained 0.50 mol phosphate per mol myosin in control muscles which increased to 0.90 with tetanic electrical stimulation. Intra-arterial injection of 1 n mol of isoproterenol increased cyclic AMP and phosphorylase a formation but had no effect on light chain phosphorylation. The results have indicated that phosphorylation of myosin can be stimulated in skeletal muscle in vivo in response to contractile activity. Similar results were reported in frog muscle by Barany and Barany (1977).

At present there is no direct evidence that the RLC of skeletal muscle are involved in Ca^{2+} regulation. In fact phosphorylation or removal of the RLC results in no change in the actin-activated myosin ATPase activity

(Weeds and Lowey, 1971). The only regulatory role of Ca^{2+} appears to be via its binding to troponin. Indirect evidence has been provided by Lehman (1978), who has demonstrated that skeletal muscle myosin can be activated by pure actin in a Ca^{2+}-dependent fashion when assayed at 120 mM NaCl. Considering the weak binding of Ca^{2+} by myosin as compared to troponin the possible significance of a potential myosin regulated system in skeletal muscle remains to be evaluated.

C. Non Muscle Myosin

The model system for investigation of non muscle myosin has been the human platelet. Human platelet myosin is similar to other non-muscle myosins, has a molecular weight of 460,000, and is composed of two heavy chains (200,000) and two different light chains (20,000 and 15,000) (Adelstein and Conti, 1972). The 20,000 dalton light chain can be phosphorylated and the phosphorylation results in increased actin-activated myosin ATPase, and an increase in the maximum isometric tension (Lebowitz and Cooke, 1978). Dephosphorylation decreases this activity (Adelstein et al, 1976). However the kinase catalyzing this phosphorylation is not Ca^{2+} sensitive (Daniel and Adelstein, 1976, Adelstein et al, 1977). It therefore appears that Ca^{2+} is not involved in the regulation of non muscle myosin.

8. Possible Involvement of Protein Modulator in Troponin

The regulation of skeletal muscle actin-activated myosin ATPase is thought to involve components tropomyosin and troponin. Troponin is composed of three subunits troponin-T, troponin-I, and troponin-C. The homology of the physical and chemical properties of troponin-C and protein modulator was initially reported by Wang et al (1975). Subsequently Dedman et al (1977a) have examined the possible functional homology of troponin-C and protein modulator, and have demonstrated that troponin-C can substitute for protein modulator in the activation of phosphodiesterase although at 600 fold the concentration of protein modulator Dedman et al (1977a) have also reported that protein modulator can form a complex with TN-I and TN-T (TN-T—TN-I—PM) and therefore substitute for TN-C. This hybrid complex was found to inhibit the actin-activated myosin ATPase activity of the reconstituted system 50% in the presence of Ca^{2+} and 20% in the absence of Ca^{2+}. In contrast the native troponin had little effect on the actin-activated myosin ATPase activity in the presence of Ca^{2+} but when Ca^{2+} was absent, the enzyme activity was decreased to 60%. These results suggested the existence of functional differences between native troponin and the hybrid complex.

Amphlett et al, (1976) have also reported that

protein modulator can substitute for TN-I and form a TN-T—TN-I—PM complex. Of interest was the observation that the protein modulator also possessed the property of interacting with troponin I to form a TN-I—PM complex which in the presence of tropomyosin could restore Ca^{2+}-sensitivity to the reconstituted actomyosin ATPase of skeletal muscle. The TN-C—TN-I complex was capable of restoration of only a small degree of Ca^{2+} sensitivity. It therefore appears that protein modulator can form functional complexes with TN-I or with TN-I and TN-T.

The substitution of TN-C by protein modulator appears to have doubtful *in vivo* significance, in muscle cells, It has been suggested however that protein modulator might have a role in the regulation of non-muscle contractile systems, similar to the role of troponin-C in muscle cells. As yet no direct evidence in favor of this suggestion has been reported.

9. Protein Modulator Regulated Glycogenolysis

Meyer *et al* (1964) first postulated that Ca^{2+} might couple muscle contraction and glycogenolysis. This theory was based on the observation that EGTA, a Ca^{2+} chelating agent was a potent inhibitor of phosphorylase kinase. Subsequently, the Ca^{2+} concentration required for

half-maximal activation of phosphorylase kinase was determined by Ozawa et al, (1967) to be about 10^{-7} M Ca^{2+}.

Equilibrium binding studies have established that phosphorylase kinase binds Ca^{2+} with high affinity. Brostrom et al, (1971) determined that at low ionic strength and in the absence of Mg^{2+} two classes of Ca^{2+} binding sites exist on phosphorylase kinase with K_d 0.3 X 10^{-7} M and 3 X 10^{-6} M. More recently Kilimann and Heilmeyer (1977) reported the existence of a single class of Ca^{2+} binding sites, K_d (Ca^{2+}) of 5.5 X 10^{-7} M capable of binding 8 mol Ca^{2+} per mol phosphorylase kinase at an ionic strength of 0.10 M NaCl. When 20 mM Mg^{2+} was included at constant ionic strength two classes of Ca^{2+} binding sites were reported, a high affinity class binding 8 mol Ca^{2+} with K_d (Ca^{2+}) of 3.0 X 10^{-6} M and a second lower affinity class binding 4 mol Ca^{2+} with K_d (Ca^{2+}) of 3.5 X 10^{-5} M. The K_d (Mg^{2+}) was determined to be 1.7 x 10^{-3} M. It therefore appears that in the presence of Mg^{2+} the phosphorylase kinase holoenzyme binds 12 mol of Ca^{2+}.

Several studies have suggested that Ca^{2+} stimulates phosphorylase kinase activity by affecting the interaction between phosphorylase kinase and its substrate phosphorylase. Heilmeyer et al, (1970) reported that the Km for phosphorylase b decreased approximately 25-fold when the concentration of Ca^{2+} was increased from about

10^{-8} to 10^{-5}M. In support of this work Gergely et al, (1975) found that the protein complex between phosphorylase and phosphorylase kinase was dissociated by the addition of EGTA.

Cohen (1973) and Hayakawa et al (1973) have established that phosphorylase kinase is composed of three types of subunits termed α, β and γ which have molecular weights of 145,000, 128,000 and 45,000 respectively. Since the native enzyme has a molecular weight of 1.28×10^{-6} daltons the subunit structure has been proposed to be $(\alpha\ \beta\ \gamma)_4$. Cohen et al, (1978) have recently reported the existence of a fourth subunit of phosphorylase kinase termed the δ subunit. This subunit was identified as the protein modulator. Unlike the Ca^{2+}-dependent interaction between protein modulator and phosphodiesterase, the protein modulator could not be dissociated from phosphorylase kinase by gel filtration in the absence of Ca^{2+}. This tightly bound protein modulator was reported in near stoichiometric amounts with the other three subunits of phosphorylase kinase (0.7 mol/mol subunit). This would suggest 3 mol of protein modulator per mol of phosphorylase kinase holoenzyme. Assuming 4 mol of Ca^{2+} bound per mol of protein modulator this would result in the binding of 12 mol of Ca^{2+} per mol of phosphorylase kinase; a value in agreement

with the Ca^{2+} binding studies of Kilimann and Heilmeyer (1977). The observation that exogenous protein modulator is capable of stimulation of phosphorylase kinase activity (Cohen et al, 1978) has been interpreted to suggest that protein modulator may also interact with phosphorylase kinase by a Ca^{2+}-dependent and reversible mechanism. The stoichiometry of interaction of this "loosely" bound protein modulator with phosphorylase kinase has not been determined. As will be presented in the Results section, the effect of modulator protein on phosphorylase kinase has also been observed in our laboratory.

10. Possible Protein Modulator Regulation of Mitosis

Using immunofluorescence technique Welsh et al, 1978 have shown that protein modulator decorates the stress fibers of non-muscle interphase cells. As the cells enter prophase, the distinct cytoplasmic localization of the protein modulator disappears commensurate with the dissolution of the cytoskeleton, and the protein modulator assumes a random distribution. At prometaphase it is localized in association with the half-spindles of the mitotic apparatus, and through metaphase and most of the anaphase the protein modulator remains localized between the chromosomes and the poles of the spindle. During the late anaphase this protein is found in the interzone region but rapidly condences into two small regions one on each side of the

midbody that separates the daughter cells. The protein modulator was not localized in the cleavage furrow during telophase. Based on these results Welsh et al, (1978) have suggested a possible role of protein modulator in the mediation of the Ca^{2+} effects on the mitotic apparatus and hence chromosomal movement.

Fujiwara and Pollard (1976) have used indirect immunofluorescence to demonstrate myosin-specific fluorescence in the cleavage furrow but not the spindle. These results and others have suggested the role of actomyosin during mitosis might be cytokinesis. The observation that protein modulator is not localized in the cleavage furrow would appear to preclude a role of protein modulator in association with actomyosin. Alternately, protein modulator could be involved with the Ca^{2+} dependent inhibition of microtubule assembly (the depolymerization of kineticope-associated microtubules is necessary for chromosomal movement to the poles). The distinctive localization of protein modulator in the mitotic half-spindle where microtubule depolymerization occurs during anaphase chromosome movement has further suggested an involvement of protein modulator in the control of the state of microtubule assembly during mitosis.

The Ca^{2+}-dependent regulation of microtubule

assembly-disassembly by the protein modulator has been studied *in vitro* by Marcum *et al*, (1978). It was determined that stoichiometric concentrations of protein modulator both inhibited and reversed microtubule assembly in a Ca^{2+}-dependent manner; in the absence of protein modulator Ca^{2+} (10 μM) caused only a slight reduction in polymerization whereas in the presence of both Ca^{2+} and the protein modulator inhibition of microtubule assembly was observed. Troponin-C was found to also inhibit and reverse microtubule assembly. The biochemical and immunofluorescent localization results therefore provide evidence for the Ca^{2+}-dependent regulation of microtubule assembly by protein modulator.