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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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