Investigation of the effects of Calmodulin and Calmodulin Binding Proteins on the steady state of actin polymerization

by

Lois C. Schwarz

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SUMMARY

The purpose of the present study was to investigate the effects of CM and/or the CMBPs on the steady state actin polymerization in the presence and absence of Calcium. It was hoped that greater insight would be obtained about the regulation of cellular mobility. It was also considered if new functions could be accredited to the CMBPs.

Viscosity and polyacrylamide urea gel electrophoresis were used to determine the possible effects of CM and/or the CMBPs. No effect was found for any of of these proteins on actin polymerization either in the presence or absence of Calcium.

ABBREVIATIONS

ABP	: Actin Binding Protein
BME	: Beta-mercaptoethanol or 2-mercaptoethanol
cAMP	: Adenosine 3':5'-cyclic-monophosphate
CB	: Cytochalasin B
CD	: Cytochalasin D
cGMP	: Guanosine 3':5'-cyclic-monophosphate
CM	: Calmodulin, activator, Ca^{+2}-dependent regulator, protein modulator, Ca^{+2}-dependent activator
CMBP-I	: Calmodulin Binding Protein I, modulator binding protein, inhibitor protein, calcineurin
CMBP-II	: Calmodulin Binding Protein II, heat stable inhibitor
CMBP's	: Total calmodulin binding protein batch
DEAE cellulose	: Diethylaminoethylcellulose
E	: Enzyme
EGTA	: Ethyleneglycol-bis-(beta-aminoethyl ether)N,N'-tetraacetic acid
ETS	: Erhlich Ascites Tumor Cells
F-actin	: Filamentous actin
FDP	: Fructose 1,6-diphosphate
G-actin	: Globular, monomeric actin
HMM	: Heavy meromyosin
HMM-SI	: Heavy meromyosin - subfragment I
MAPs	: Microtubule associated proteins
MF	: Microfilament
ml	: millilitre
mM	: millimolar
MT	: Microtubule
NAD	: Nicotinamide adenine dinucleotide
ng	: nanogram
nm	: nanometre
PDE	: Calmodulin dependent cyclic nucleotide phosphodiesterase
SDS	: Sodium dodecyl sulphate
Tm	: Tropomyosin

Tn	: Troponin
Tn-C	: Ca^{+2} subunit of troponin
Tn-I	: Inhibitory troponin subunit
Tn-T	: Tropomyosin binding subunit of troponin
uM	: micromolar
UV	: ultraviolet

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Chapter I

INTRODUCTION

The purpose of the present study was to investigate the effects of calmodulin (CM) and the calmodulin binding proteins (CMBP's) from a bovine brain extract on the filamentous state of skeletal muscle actin. Viscosity measurements and SDS-urea acrylamide gel electrophoresis were used to determine whether interactions between these proteins were taking place. The reasons for exploring the effects of CM on actin polymerization were basically threefold.

First, both CM and actin are ubiquitous among eucaryotic organisms. Although the presence of both proteins in many tissues does not preclude that they function together, in at least one case, these two highly conserved species have been found to co-exist. Means and Dedman (1980), as well as other researchers, have indicated the presence of CM on actin stress fibres in resting cells using immunoflourescence. An obvious question arising from this observation is why should CM be found in such close proximity to actin? Perhaps it is controlling actin-related phenomena such as polymerization.

However, actin does not always exist as these highly ordered stress fibres in non-muscle cells or as the well organized fibres of skeletal muscle. Cells preparing for cell

division have been observed to contain their actin in a widely dispersed fashion throughout the cell. How does actin suddenly transform from a highly organized lattice network into a dispersed, unintegrated body of protein? What confers such flexibility to the contractile protein? At least in the case of gelation (i.e. the cross-linking of F-actin polymers), Ca^{+2} and various "non-contractile" gelating proteins have been found to control the organizational state of F-actin and in some cases, that of G-actin.

These observations have led to the second reason for investigating the interaction of these two proteins. Wang and Waisman (1979) have outlined the Ca^{+2} regulation of many cellular processes by CM, such as: cyclic nucleotide metabolism, glycogen metabolism, some contractile activities, and microtubule assembly. In light of its seemingly universal role in regulating Ca^{+2} related phenomena, it was questioned if CM was also regulating actin activities which may depend on Ca^{+2}.

One actin activity which has been shown to be indirectly influenced by the presence of CM and Ca^{+2}, is the activity of the myosin ATPase of smooth muscle and platelets. In these systems, CM activates a myosin light chain kinase which then phosphorylates the 20000 dalton light chains of myosin. Upon phosphorylation, there is an increased actin activation of the myosin ATPase activity. However, before actin can perform this function, it must exist in a polymer-

ized form. Does CM also affect the ability of actin to polymerize and thereby alter its activation of myosin ATPase - a well known Ca^{+2} dependent process? Does CM alter actin polymerization and its resultant ability to form cytoskeltons or induce movement in single-celled organisms? In short, we sought to investigate whether CM exerted a direct influence on actin as well as its well known regulation of myosin activity.

The third reason for testing whether there was an interaction was based on CM's similarity to troponin-C, the Ca^{+2} binding subunit of troponin. In skeletal muscle, troponin-C is responsible for the Ca^{+2} sensitivity of the contractile process in which actin is involved. Furthermore, troponin-C is considered to be homologous to CM and has been shown to bind Ca^{+2} as did CM. Troponin-C, in association with Tn-T or Tn-I, has also been shown to be associated with actin. Therefore, does CM associate with actin in non-muscle cells where Tn-C apparently does not exist? It also remains to be considered whether the CMBPs would act in concert with CM to associate with actin as the various components of the troponin complex and Tm are required for association of Tn-C with F-actin (Ishiwata, 1978).

It was also questioned whether the calmodulin binding protein(s) would affect the polymerization process, or reverse the effects, if any, of CM on this phenomenum. The reasons for this query were that CMBP's have been shown to

reverse the effects of CM on many of its activated enzymes occur in the case of actin polymerization. Also, to date, no known function of CMBP-I has been reported except to bind CM. This is contrary to other proteins which bind CM since most have been reported to have other activities, e.g. phosphorylase kinase or myosin light chain kinase (Wang, et al 1980). Therefore, another objective of this present study was to investigate whether or not the control of actin polymerization may be a putative function for this protein.

Chapter II

LITERATURE REVIEW

2.1 THE FUNCTIONS OF ACTIN IN NON-MUSCLE CELLS

In 1943, Straub illustrated that the "contracting myosin threads", as they were termed at that time, were in fact, myosin filaments associated with another protein in skeletal muscle. To denote the "new protein", Straub coined the term ACTIN (Straub 1943). About 10 years later, the proteins actin and myosin were proposed to be located in separate filaments of defined length in striated muscle. Hanson and Huxley (1953) proposed that these filaments were organized into discrete, overlapping arrays which produce a distinct banding pattern. The evolution of the "sliding filament mechanism" for force generation during contraction began with these ultrastrucural observations, and is now widely accepted (Huxley 1976). Since the discovery of actin in muscle cells of all types and the elucidation of its role in muscle contraction, actin has been found to be an ubiquitous protein among eucaryotic tissues.

Actin was first isolated from non-muscle sources in 1952 when it was isolated from the slime mold *Physarum polycephalum* (Loewy 1952). Shortly after, studies on the role of actin were carried out with the general conclusion that actin

was involved in the cytoplasmic streaming of this organism's giant plasmodia. It is now thought that a wide range of phenomena involving actin can be studied with this organism including cell shape changes, translocation of cells along surfaces, and protoplasmic streaming (Jacobson 1976).

Actin has also been found in another slime mold, Dictyostelium discoideum. Spudich and Cooke (1975), regenerated, in vitro, actin filament nets similar to those seen at the migrating edges of cells and filament bundles similar to stress fibers also seen in vivo (Wessells, et al 1973). Observations on these systems have led to the conclusion that actin may be involved in producing movement as well as in maintaining cell shape via stress fibres.

Despite the usefulness of these observations in assigning various roles for actin such as maintaining shape, producing cytoplasmic streaming, mediating shape change and mediating translocation in cells along surfaces, it was still necessary to illustrate directly the relationship between actin and the various cellular activities. Even when the properties of isolated contractile proteins have been determined, and actin has been localized in fixed cells, it does not necessarily prove that these molecules cause movement or maintain shape. In order to begin to show such relationships, model systems for motility in living cells had to be developed. Cellular extracts have been important in research finding the roles of actin in non-muscle cells, be-

cause these model systems could be manipulated experimentally.

The ability of cytoplasm to move even after outer membranes have been removed was first shown by Allen, et al (1960). In further characterizing this phenomena, Thompson and Wolpert (1963) illustrated the temperature dependence and ATP stimulation of the cellular extracts in Amoeba proteus. Pollard and Ito (1970) showed that a viscosity change took place when A. proteus extracts were warmed. This change was attributed to actin polymerization. They also made the important observation that movement in this cell-free system occurred only when this polymerized actin and thick filaments, thought to be myosin, were present. It was, therefore, illustrated that filamentous actin was essential for movement in the model using cellular extracts. Shortly thereafter, it was shown that movement also depended on a free Ca^{+} concentration being greater than a threshold concentration of $7x10^{-7}M$ in the amoeba Chaos chaos (Taylor, et al 1973). Chaos chaos (Taylor, et al 1973). The early studies were important because they allowed observable movement to be controlled experimentally, thereby giving more information on the factors influencing movement. However these studies had two limitations. First, A. proteus was difficult to culture and therefore amounts of the extract available for study were limited. Second, microscopic study introduced subjectivity.

A good alternative was Acanthamoeba castellanii. It was easy to grow and its contractile proteins were partially characterized. Since actin and myosin have been implicated in motility of cells through the observations on movements of cellular extracts, the ultimate goal now was to try to substantiate the role of actin and myosin in motility by approaching the situation from the opposite direction. Do actin and/or myosin alone produce movement? Are other proteins also required?

Before these questions can be answered, some background information should be considered. When the model system is isolated, namely cold A. castellanii extracts, and allowed to warm to room temperature, actin gelation occurs followed by movement which is described as "contraction" of the gel. The gel is similar to a weak solid (Pollard, et al 1976). Electron microscopy was used to detect actin filaments and utilizing SDS polyacrylamide gel electrophoresis, actin was found to be a major macromolecular component of the gel. Some myosin and a small amount of a high molecular weight protein were also found in the gels. The gelation process is highly temperature dependent and requires only Mg^{+2} and ATP.

If the gelled extracts are left standing at room temperature, they will "contract" and this was referred to as "movement" in the earlier studies. The contracted gel consists of virtually all the cell's actin, myosin, and the

high molecular weight protein. The rate of contraction can be monitored by turbidity and the various ionic factors influencing this rate can be determined. Low concentrations of Ca^{+2} ($1x10^{-8}M$ to $4x10^{-7}M$) lead to the greatest rates of contraction. These experiments answered the second question, namely, that other proteins as well as actin and myosin are needed for movement, i.e. contraction.

Lastly, the actin component of the contracted gel was isolated in an attempt to answer the first question, i.e. can actin alone cause formation and contraction of a gel and is it implicated in causing movement in vivo. In fact, it was found that purified actin formed a solid gel with $MgCl_2$, ATP, and KCl when warmed to room temperature (Pollard, et al 1976). The concentrations of the various ions and that of actin were all physiological giving credence to the idea that this may be occurring in vivo. Pollard proposed that this gel may be the important cytoskeletal component which gives the cytoplasm its semisolid consistency. Phase contrast microscopy illustrated that the cortical cytoplasm has the same appearance as the gelled extract. Electron microscopy has shown that the cortical cytoplasm of glycerinated cells is similar to assemblies of actin filaments (Pollard, et al 1976). Although these experiments suggest a role for actin in cytoskeletal formation, the question remains as to how it is functioning in movement. To this end, the studies performed on the A. castellanii extracts were the most re-

vealing. They implicated the involvement of actin and myosin and suggested that Ca^{+2} may regulate motility since it controlled the rate of contraction of the gels (Pollard, et al 1976).

More recently, it has been shown that the gelled extracts containing actin and myosin were non-motile with Ca^{+2} concentrations less than the micromolar range. It was suggested that solation (liquefying) of the gel may be required for it to contract (i.e. move). This contraction required greater than micromolar concentrations of Ca^{+2}. From these observations, it was suggested that solation was required for movement (Solation-Contraction Coupling Hypothesis) whereas gelation may be required to form the cytoskeleton (Condeelis, et al 1979). It was suggested that gelation requires the presence of other proteins which would be expected to have three properties :

1. In the presence of millimolar ATP, remain bound to actin.
2. Inhibit the interaction between actin and myosin.
3. Inhibit gelation activity with micromolar concentrations of Ca^{+2}.

A 120K dalton protein factor was found which filled the above criteria. Condeelis concluded that the regulation of gelation by micromolar concentrations of Ca^{+2} <u>in vitro</u> may be the mechanism by which actin can perform two functions:

1. form the cytoskeleton

2. produce force (Condeelis, et al 1979).

The acrosomal reaction of invertebrate sperm, such as in Thyone sperm, has been studied as another specialized experimental model under which to consider the role of actin. Tilney (1978) showed that actin was involved in mediating cell shape change in this system. The reaction is thought to involve a rapid polymerization (10 seconds) of actin resulting in a process which can exceed 90 micrometers in length. Tilney (1979) is presently resolving the details of this phenomena. In this system, actin is thought to mediate the change in the shape of the cell by producing a process which is capable of puncturing an egg for sperm penetration. It is interesting to note that myosin does not seem to be required, but rather the polymerization of actin itself is sufficient to mediate puncturing.

Other examples of the function of actin in non-muscle cells can be noted by considering its function in higher eucaryotic organisms. The first convincing demonstration of actin in vertebrate non-muscle cells was performed by Bettex-Galland and Luscher (1959) when they isolated a crude preparation of actin and myosin from human platelets. The platelets have proven to be an excellent model system because actin exists in a relatively high concentration (approximately 20-30% of the total cell protein), and therefore is relatively easier to isolate and characterize than actin from other cells. Actin has been implicated through bio-

chemical and structural evidence to be important in the shape change acompanying platelet activation and therefore may be performing some type of cytoskeletal role. Platelet actin has also been implicated in clot retraction and support for this role has been obtained by examining both the geometry of the activated platelet and the internal arrangement of its actin and myosin (Adelstein 1978). Actin has also been implicated in platelet secretion where contraction of the actin filaments could be involved in the transport of secretory vesicles to the cell surface. Some support for the involvement of the contractile nature of actin in this process came from data showing that there is an increase in myosin light chain phosphorylation, a biochemical phenomena, which was correlated with an activation of the contractile machinery (Daniel, et al 1977).

Secretion has also been implicated as a role for actin in other secretory tissues such as the pancreas and the liver. Within the beta cells of the pancreas, there is a filamentous web just below the plasma membrane which is thought to be composed of actin (Howell and Tyhurst 1979). They found that if the web was disrupted with the fungal metabolite cytochalasin B, there was an enhanced insulin secretory response to glucose. Therefore, they suggested that the actin filament web below the cytoplasm membrane may be impeding the movement of the vesicles. F-Actin was shown to bind the insulin storage vesicles and the strength of this interac-

tion could be decreased in the presence of Ca^{+2}. They also noted that the Ca^{+2} concentration does in fact increase in the pancreatic cells upon stimulation of insulin release which suggested that Ca^{+2} may be preventing the interaction between F-Actin and the storage vesicles in vivo. However, further investigation is required. Similar types of experiments were carried out with isolated rat hepatocytes and it was found that phalloidin and cytochalasin D inhibits lipoprotein secretion and that these effects may have resulted from a modification of actin microfilament function since both of these drugs have a high affinity for actin (Prentki, et al 1979).

Secretion as well as other activities have been implicated as a function for actin in brain. The particular secretory process in this case is that of neurotransmitter release via exocytosis. Berl, et al (1973) proposed a hypothesis for the release of acetylcholine by a mechanochemical transduction between presynaptic vesicles and membranes. This hypothesis has been advanced for the general phenomena of exocytosis (Hoffmin-Berling 1956) and relies on the basic model in the actomyosin system of muscle for the conversion of chemical energy to mechanical energy. The model proposed that a torsional interaction between brain myosin associated with the vesicles and the brain actin of the synaptic membrane (in the presence of Ca^{+2}) could produce a conformational change in the vesiclar membrane which

would cause an opening of the membranes for the release of neurotransmitter. To terminate the action, Ca^{+2} would be removed and consequently the ATPase activity would decrease and loss of torque would occur (Berl, et al 1973).

This hypothesis, where actin and myosin are thought to be involved in neurotransmitter release, would be more attractive if the involvement of Ca^{+2} in the interaction could be substantiated since Ca^{+2} was found to be a requirement for the similar process in muscle. Ca^{+2} had already been proven necessary for neurotransmitter release by Katz and Miledi (1965). As an initial piece of evidence for the involvement of Ca^{+2}, Manhedran, et al (1974) found a Ca^{+2} sensitive component which could be isolated from the actomyosin complex from brain. Puszkin, et al (1974), showed that the synaptic vesicles had a Ca^{+2}-Mg^{+2} dependent ATPase activity. The Mg^{+2}-ATPase activity was Ca^{+2} sensitive after a Ca^{+2} sensitive component was added to the actomyosin. Vesicle release was Ca^{+2} dependent. These facts suggested that the presence of Ca^{+2} may be triggering the contractile machinery for neurotransmitter release in brain as it triggers the contractile machinery of muscle for a contractile event.

Actin has also been implicated in other activities in the brain such as forming functional connections between the plasma membrane and the nucleus. Using electron microscopy, Metuzals, et al (1974) noted that a filamentous network extends from the synaptic membranes to the nuclear pores. They

proposed that this network may serve a transducing function in linking the events at the synaptic membrane to the genetic code of the neuron. Exactly how actin would perform this function is unknown.

Another example of the activity of actin in brain came from studies on axonal transport, the process involved in carrying proteins from the neuronal cell body to the axons and synaptic terminals. According to the hypothesis developed by Hoffman and Lasek (1976), microfilaments (polymerized actin) continually emanate from the site of synthesis in the cell body and move toward the synaptic bouton where they are finally disassembled. The model for the mechanism by which microfilaments are involved in axonal transport was based on the observations that myosin is associated with the actin and therefore the possibility for force generation exists via the hydrolysis of ATP. Actin also appears to be attached to the lateral sides of the inner surface of the axolemma and this is proposed to be the site of force generation. The model proposes that radial force generated through concerted interactions at the axolemma could be transferred to linear force generation with resultant cytoskeletal movement.

Lastly, in connection with the brain, actin has also been implicated in neuronal growth. Levi-Montaleini, et al (1979) noticed that nerve growth factor (NGF) binds to receptors on the nerve plasma membrane and shortly thereafter,

the protein constituents of immature sympathetic nerves are greatly altered. The actin filaments (microfilaments) were found to fill the space between the cell nucleus and the cell membrane as do the microtubles. They proposed that these filaments provide a strucural foundataion for the growth of the nerve axons and may provide the propulsive force for nerve elongation. They also proposed that NGF controls the assembly of actin by inducing rapid and massive nucleation so that elongation can occur spontaneously. The fact that fungal metabolites such as cytochalasin B block neurite growth, and also prevent actin polymerization is further evidence that actin is involved in neuronal elongation (Tamada, et al 1971). Not only does nerve growth factor potentiate actin polymerization, but it also favors formation of actin paracrystalline structures which activate the myosin ATPase to a greater extent than actin alone (Calissano, et al 1978). This may provide more energy for the elongation process, however exactly what is occurring <u>in vivo</u> is not known.

Actin is involved in many processes in several cells, and this is not surprising in view of the fact that it is thought to be the second most abundant protein in nature (Ellis 1979). It is not possible to cover the involvement of actin in all tissues, however extensive reviews and published symposia have been written on the general involvement of actin in various cells (Clark, et al 1977; Hitchcock

1977; Goldman, et al 1976; Perry, et al 1976; Adelstein, et al 1979). After noting the role of actin in the various cells mentioned, a general picture emerges illustrating that actin is likely serving two functions in the cell. The first activity of actin is to form a cytoskeleton as in all the single celled organisms and eucaryotic tissues mentioned. The second function is to interact with myosin ATPases which generate force for contraction and thereby produce gross movements as in muscles or subtle creeping movements of single celled organisms such as P. polycephalum, D. discoideum, and A. castellanii. Internal force requiring movements may also be the result of the interaction between actin and myosin such as in the secretory activities of platelets, pancreas, liver and brain or as in axonal transport in brain.

In order for actin to carry out its various activities, the state of its filamentous structure is important. In performing a cytoskeletal function, the actin is thought to be highly dynamic and flexible so that it can change from a highly ordered state, as in the stress fibers of resting cells, to a diffuse non-filamentous state as in cells preparing for division. Furthermore, only F-Actin is capable of activating myosin ATPase. Since the state of actin polymerization is so important with respect to its various activities, the polymerization reaction itself will be discussed in some detail.

2.2 THE POLYMERIZATION OF ACTIN

The following section deals with the first of the mentioned activities of actin, namely its polymerization. Before the regulation of actin polymerization by Ca^{+2}, can be outlined, Ca^{+2} binding proteins and other proteins are described, some background on the polymerization reaction itself is presented.

Actin has been shown to polmerize in two steps. The first reaction is called a nucleation reaction and is rate limiting. Kinetic data implies that this reaction is third or fourth order suggesting that three or four actin monomers must aggregate to form a 'nucleus' (Asakura and Oosaua 1960; Asakura, et al 1960; Oosawa, et al 1961; Kasai, et al 1962; Oosawa and Kasai 1962; Asakura, et al 1963; Kasai 1969). The second step is the elongation of the nucleus to form long polymers of actin and is generally thought to be first order. When actin monomers aggregate during polymerization, one molecule of ATP is hydrolyzed per monomer to form polymers of actin bound ADP.

The kinetics of the polymerization process have been studied in much detail by Wegner (1976). His experiments have shown that actin monomers most likely associate and dissociate from both ends of the nucleus but the rate constants for the two ends of the growing filament are probably different. Since the equilibrium constants are related to the rate constants, they are also different for the two ends

of the molecule when ATP is present. The equilibrium constants or "critical concentrations" represent the concentration of monomer in equilibrium with polymer as seen below:

$$(\text{actin})_{N+1} \;\langle===\rangle\; \text{actin}_{N} + \text{actin}.$$

Wegner has extended the investigation to show that the growing filament has a net polymerizing end and a net depolymerizing end. From these observations, he proposed that actin grows in a head to tail fashion, i.e. actin grows at one end and shortens at the other. Further evidence for this view has recently been published (Brenner, et al 1979).

The model states that under polymerizing conditions, there would be a net polymerizing end with more protomers being added at this end and less monomers being lost at the depolymerizing end. However, conditions could exist during polymerization where both ends are polymerizing.

Polymerization ceases, and steady state for the system as a whole is achieved, when the net rate of addition of monomers at the polymerizing end equals the net rate of loss at the depolymerizing end. Under steady state conditions for the system as a whole, neither end of the filament will be in equilibrium since their equilibrium constants or critical concentrations are different.

During depolymerization, there is a greater loss of monomers from the net depolymerizing end than is gain of these monomers from the net polymerization end. However, conditions could also exist where depolymerizing actin may have two depolymerizing ends per filament.

2.2.1 Physical factors affecting the polymerization of Actin

Two main physical factors were shown to influence the ability of actin to polymerize or in other terms, which change the critical concentration. These are the temperature and solvent conditions (Gordon, et al 1976). By varying these conditions, it is possible to determine which factors provide the optimum environment for polymerization and it is also possible to determine which of these factors will influence nucleation and/or elongation. At 25°C and 1 mM $MgCl_2$, the polymerization of 0.3 mg/ml of actin from both A. castellanii and skeletal muscle was very slow. If sonicated F-actin was added to the reaction mixture to serve as nuclei, polymerization rapidly increased. Since polymerization proceeded once the nuclei were added, Gordon explained that it was the nucleation reaction which was limited under these conditions. However the rate of nucleation could be increased by increasing the $MgCl_2$ concentration to 5 mM illustrating therefore the requirement of $MgCl_2$ for nucleation. It follows then, that at 0.1M KCl and 25°C, nucleation again was rate limiting since there was no $MgCl_2$. Gordon showed that once nuclei were added, polymerization increased markedly with a rate of elongation comparable to that in the presence of Mg^{+2}.

Gordon also demonstrated that by decreasing the temperature to 5°C, the rate of nucleation could be decelerated even in the presence of 5 mM $MgCl_2$. By adding nuclei, the

rates of polymerization were comparable to those obtained at 25°C. Therefore the temperature effect was largely on nucleation. Utilizing 0.1M KCl at 5°C, nucleation was again slowed and polymerization proceeded at a comparatively higher rate once nuclei are added when using muscle actin. A. castellanii actin failed to polymerize even after the addition of nuclei. Since A. castellanii actin can polymerize in 0.1M KCl at 25°C, especially after the addition of nuclei, the decreased temperature must have been affecting elongation as well as nucleation.

Through various viscosity studies, Gordon found that in 2 mM $MgCl_2$, the viscosities of A. castellanii and muscle actins were the same at all concentrations at 25°C and 5°C. He suggested that the lack of temperature dependence implied that the enthalpy of polymerization under these ionic conditions was small, and that the polymerization may be driven by entropy.

These authors have extended their investigations by comparing the polymerization characteristics of actin from a variety of sources. The critical concentration was the variable most often considered, and it was determined under a variety of ionic and temperature conditions (Gordon, et al 1977). They also concluded that the polymerization properties of the vertebrate non-muscle actins (platlet, brain, and liver) and A. castellanii actin were qualitatively similar to the polymerization of rabbit skeletal muscle actin,

but quantitativly the non-muscle actins were more similar to each other than to muscle actin.

It was noted that the extracts of non-muscle tissues contained monomeric G-actin at concentrations that were much greater than the critical concentrations of the purified actin. From these observations, it was suggested that various actin binding proteins may be preventing the polymerization of the actin in the cell extracts or in vivo (Gordon, et al 1977). In fact, to date, many actin binding proteins have been isolated from many different tissues and investigators are now attempting to determine if and how these proteins may be functioning in the cell to regulate the state of actin polymerization.

2.2.2 Effects of Actin Binding Proteins

Since the concentration of G-Actin was well above the critical concentration for its polymerization in vivo, it was soon suspected that there were factors present in the cell which were preventing or inhibiting actin polymerization. It was not long before many actin binding proteins were discovered, some of which were found to inhibit actin polymerization, while others were in fact, found to enhance this phenomena. Some proteins were also found to bind F-Actin and cross-link the filaments in a process called gelation.

2.2.2.1 Profilin

Profilin (molecular weight 16000 daltons) was first isolated from spleen (Carlsson, et al 1976) but a wide distribution of this protein is suspected since it is considered to be present in some invertebrate sperm as well (Tilney, 1975). Carlsson, et al (1976), characterized the protein and demonstrated that it formed a 1:1 complex with G-Actin and that this complex was resistant to polymerization. Later this resistance was shown to exist under a wide range of pH and salt concentrations (Lindberg, et al 1979) and only after mild treatment with trypsin was the actin released. The liberated actin was shown to maintain its polymerization characteristics.

2.2.2.2 DNase I and 5'-nucleotidase

Another protein which binds G-Actin is DNase I. DNase I (molecular weight 33000 daltons) was first reported to exist as a complex by Lindberg (1967) when it was isolated from calf spleen. It was later shown by Lazarides and Lindberg (1974) to be a specific actin binding protein. Mannberg, et al (1975), illustrated that DNase I directly affected the polymerization of actin in the presence of ATP by forming a 1:1 complex with actin. Furthermore, DNase I competitively displaced HMM-SI and tropomyosin individually from the actin polymer. Mannherz, et al (1975), also showed that in the presence of ATP, the DNase I-actin complex was dissociable

when the actin to HMM-SI molar ratio was 1:2 provided tropomyosin was present. DNase I, in addition to forming a 1:1 complex with G-Actin, also caused the depolymerization of F-Actin resulting in the formation of 1:1 complex. Troponin and tropomyosin were found to slow down but not stop the depolymerization process. In the absence of ATP, heavy meromyosin (HMM) completely protected F-Actin against DNase I. However in the presence of ATP, HMM dissociated from F-Actin, F-Actin depolymerized, and DNase I activity was inhibited by binding to actin (Hitchcock, et al 1976). Further characterization of this complex has been undertaken more recently by Rhor and Mannherz (1978). These authors identified the natural occurrence of a protein in bile which was able to reactivate the naturally occurring DNase-Actin complex of rat secretory pancreatic juice or synthetic actin-DNase I complex. The protein was identified as 5'-nucleotidase and later the effects of this enzyme on actin polymerization were considered (Mannherz and Rhor, 1978). Upon investigating the effects of 5'-nucleotidase on the DNase I-actin complex, it was found that the DNase degrading activity could be fully reestablished in the presence of 5'-nucleotidase. However the ability of actin to polymerize could only be recovered if phalloidin was also present. The exact mechanism as to how 5'-nucleotidase achieved this reversal of DNase inhibition was not determined but some additional information was obtained on the

protein interactions taking place. It was found that the DNA degrading activity in the presence of 5'-nucleotidase was strongly dependent on ionic conditions, the molar ratio of actin to DNase I, and temperature. An increase in DNase activity was greatest when actin polymerization was favoured (i.e. when the concentration of KCl = 0.1M). If phalloidin was present under these ionic conditions, the optimum actin-DNase I:5'-nucleotidase molar ratio for releasing the DNase I was 3:1. The fact that the state of actin polymerization was important to the functioning of 5'-nucleotidase suggested that there may be an interaction between these two proteins. This suggestion was further substantiated by their unpublished observation that 5'-nucleotidase accelerated the rate of actin polymerization, a phenomena known to depend on the interactions of proteins. It was proposed that DNase I activity increased in the presence of 5'-nucleotidase, because actin bound to 5'-nucleotidase and this binding then resulted in the release of DNase I from its complex with actin. The authors further speculated that since 5'-nucleotidase was a a widely distributed plasma membrane constituent of eucaryotic cells, it may act as an anchorage protein for F-Actin, thus playing a part in controlling its availability for mobile responses.

DNase I and profilin are the only proteins reported which form a "tight complex" with actin, thereby preventing polymerization into filaments. However a number of proteins

have been shown to "interact" with G-Actin, at least temporarily, to increase its polymerization and many of the same proteins also bind F-Actin to either induce or inhibit gelation (i.e. cross-linking of actin filaments to each other). Many of the proteins which have been shown to interact with F-Actin have not been tested for their ability to bind G-Actin and of its ability to polymerize.

2.2.2.3 α and β actinins and villin

The α and β-actinins have been shown to have intermediate molecular weights (subunit molecular weights 43000 and 86000 daltons respectively) and have been found to interact with G-Actin as well as F-Actin. β-actinin has been isolated from the myxomycete Physarum polycephalum (Muryama, et al 1976), and although it is of lower molecular weight than muscle β-actinin, both proteins were shown to display similar interactions with actin. They have been shown to accelerate the polymerization of G-Actin, inhibit the depolymerization of F-Actin, induce formation of shorter actin filaments and to inhibit the gelation of F-Actin (Kirkpatrick, et al 1973). It was also suggested that β-actinin may serve as a filament terminator in vivo. Despite these early predictions about the functions of β-actinin, Clarke and Spudich (1977) have questioned whether this protein was simply a dimer of denatured Physarum actin. However, it was illustrated that Physarum actinin and actin were indeed dif-

ferent proteins by Hatano and Cwaribe (1979), based on molecular weight determinations using SDS-polyacrylamide gel electrophoresis and on their varying ranges of sensitivity to ammonium sulphate. Physarum actinin precipitated in the presence of 50 to 65% ammonium sulfate whereas Physarum actin precipitated between 15-35% ammonium sulfate. Furthermore, Physarum actinin did not interact with Physarum myosin or muscle heavy meromyosin myosin. These authors further characterized the polymerization of G-Actin and found that when G-actin and actinin were mixed in a weight ratio of 1:9 and polymerization induced by adding 0.1M KCl, the viscosity was the same as control F-Actin without actinin. If 2mM $MgCl_2$ was used instead of 0.1M KCl, the viscosity increased only to 1/7 that of control F-Actin. On the contrary, if Physarum actin was first polymerized in the presence of 2mM $MgCl_2$ and actinin then added, the viscosity decreased rapidly, suggesting that the F-Actin filaments were broken into smaller segments or that F-Actin polymers had been transformed into more labile structures in the presence of actinin. It was also shown that the actinin bound to the polymers formed in the presence of $MgCl_2$ but clear evidence showing the binding of actinin to polymers formed in 0.1M KCl was not available.

Similar characteristics with respect to effects on actin polymerization have been found when comparing α and β-actinin. Both actinins accelerate the polymerization of

G-Actin and inhibit the depolymerization of F-Actin, but only α-actinin promotes the gelation of F-Actin. Much of the work with non-muscle α-actinin has been done utilizing the horseshoe crab sperm as it is found in the filamentous bundle of the acrosomal process (Tilney 1975). It should be noted however that α-actinin has also been found in cultured fibroblasts and in regions of intesinal microvilli. Some researchers have confused the location of α-actinin with another protein termed "villin" found in the microfilament core of the microvilli (Mooseker and Tilney 1975). Both proteins have similar characteristics. Villin (molecular weight 95000 daltons) is slightly larger than α-actinin, but both proteins gelate α-actin. It was suggested that villin cross-linked the microfilament core of the microvilli laterally along the length of the core and to the cytoplasmic side of the plasma membrane. Evidence for this suggestion arose from the findings that cross-linkages remain attached to the microfilament core after removal of external membranes (based on electron microscopy) and only villin is present in high enough concentrations to account for this cross-linked core structure. Also, specific labeling of the cores has been achieved with villin and actin antibodies, followed by second antibody labelling with ferritin. The anti-villin labelling led to a greater increase in diameter than anti-actin labeling, as would be expected if villin was projecting outwards laterally from the filament core

(Bretscher and Weber, 1979). Despite these similarities in cross-linking activity, they seemed to temporarily clarify the confusion by pointing out that villin and α-actinin were distinct based on slight differences in molecular weight, lack of immunological cross reactivity and slight differences in location. It should be noted, however, that the controversy over whether α-actinin and villin are similar proteins has not been resolved. In a recent paper, Carroway, et al (1980), have found that immunologically, microvillus "villin" and muscle α-actinin are indeed identical. It awaits to be seen whether these two proteins can be distinguished based on their localization in the microvillus. It was reported that villin resided in the microfilament core of the microvilli while α-actinin was found in the terminal web, based on immunoflourescence (Bretscher and Weber, 1979).

Although α-actinin may not be the cross-linking protein of the microvillus, research has indicated the presence of α-actinin in brain where it may function in a manner similar to that seen in the Z-lines of skeletal muscle (i.e. where it serves an anchoring fuction). Puszkin, et al (1976), presented evidence to show that α-actinin was present in enriched fractions of synaptic vesicles and yet absent in synaptic membrane preparations. It was speculated that the α-actinin on the synaptic vesicles could interact with actin located near the exterior or the vesicle. If actin was at-

tached to the synaptic membrane it was suggested that it could interact with α-actinin on the vesicle. Simultaneously actin could interact with myosin, resulting in a contractile event which may then move the vesicle. Whether or not this is taking place in vivo is speculative at the moment and requires further research.

2.2.2.4 α-actinin and Actin Binding Proteins (ABP)

Muscle and brain were not the only mammalian tissues to which the localization of α-actinin could be attributed. This protein has also been found in platelets and the effects of α-actinin on the organizational state of actin have been further investigated in this tissue. Schollmeyer, et al (1978), have investigated the effects of α-actinin on cross-linking α-actin (gelation) in the presence of "Actin Binding Protein (ABP)", also found in human platelets. Actin Binding Protein (molecular weight 257000 daltons) is one of a number of high molecular weight proteins which has been shown to cross-link actin. In their studies, α-actinin alone was found to randomly cross-link actin into gels in the presence of tropomyosin (Tm) and troponin (Tn). ABP alone, also caused formation of gels in actin solutions, with or without added Tm-Tn complex. However when platelet ABP and α-actinin were added sequentially to solutions of actin Tm-Tn, gelation again occurred, but the actin filaments were in orderly parallel arrays in contrast to the

random orientation of filaments produced during gelation when either ABP or α-actinin was added. Furthermore, if cytochalasin B (CB) was added to a solution of actin-Tm-Tn followed by ABP and α-actinin, a dramatic effect on filament organization occurred. Also if solutions of actin and Tm-Tn were allowed to gel in the presence of ABP and α-actinin, and then CB was added to the mixture, a dramatic effect on filament organization was again observed. Electron microscopy revealed that when CB was added first, the parallel orientation of the microfilaments was lost. However if CB was added after the cross-linking proteins, complete disruption of the filament bundles occurred. The resulting filament structures were shorter and thicker than those of the original actin-Tm-Tn gel. Upon centrifugation of this solution, α-actinin was found in the supernatant suggesting that α-actinin was important in promoting lateral filament association in actin gels. ABP was suggested to be important for promoting the desired conformation or association required for lateral alignment of filaments into parallel arrays. The data also suggested that the primary influence of CB was on α-actinin rather that ABP or the actin-Tm-In complex (Schollmeyer, et al 1978).

There has been some controversy over the exact role ABP plays in gel formation however there is little doubt that this protein is a component of the gel that forms when cold sucrose-containing extracts of rabbit macrophages are warmed

to room temperature (Hartwig and Stossel, 1975; Stossel and Hartwig 1976). Extracts of Acanthamoeba castellanii sea urchin eggs and HeLa cells have also been reported to contain a high molecular weight protein in gels formed from extracts (Hartwig and Stossel, 1975; Stossel and Hartwig, 1976). However, an equivalence among these proteins remains to be established. The controversy surrounding the role of ABP in gel formation arose from the observation of Maruta and Korn (1977) that only 3% of the gel forming activity in extracts could be attributed to ABP. The remaining 97% of the activity could be attributed to four smaller proteins and to one of these four in particular which will be discussed in a later section. Although these results seemed to dampen the importance of ABP in gel formation, it can not be ignored that cytochalasin B which disrupted microfilament organization and function in cells, also interfered wtih the interaction between actin and ABP in vitro (Hartwig and Stossel, 1976; Weihing 1976). The location of ABP in platelets was shown to be in the ectoplasmic layer just below the surface membrane. The localization of actin within this same ectoplasmic layer was used to support the contention that the disruption of pseudopod formations (which originate from the ectoplasm) by CB was due to a disruption of the actin-ABP interaction in platelets. The confusion over the role of ABP in vivo has not been resolved.

2.2.2.5 Actin Binding Protein and Filamin

Further controversy surrounding ABP originated over whether it was equivalent to filamin. Filamin is a 220000 dalton, high molecular weight protein from smoooth muscle which is thought to exist as a dimer (Shizuta, et al 1976; Wang 1977). Filamin antibodies reacted with microfilament bundles of cultured mammalian cells, with microfilaments in microspikes, with ruffling areas, with regions of cell to cell contact which did not react with anti-myosin and generally with regions of the cell which were more subject to dynamic structural alterations in microfilament organization (Heggeness, et al 1977). These observations suggested that filamin and/or ABP were associated with the actin filaments. Neither filamin nor ABP formed filaments in the absence of actin, however both proteins have been shown to bind actin filaments <u>in vitro</u> (Hartwig and Stossel, 1975; Maruta and Korn, 1977; Shizuta, et al 1976; Wang 1977; Wang, et al 1975; Wang and Singer, 1977).

Despite the similarities, there have been some reported differences in the amino acid composition of filamin (Shizuta, et al 1976; Wang 1977) and ABP (Stossel and Hartwig, 1976). These differences may have been reflecting a tissue and/or species specificity. Studies using CB have increased the uncertainty whether or not ABP and filamin are the same proteins. This fungal metabolite was shown to disrupt the ability of ABP to form gels with actin (Hartwig and Stossel,

1976; Weibing 1976) unless Tm-Tn was present before CB was added, when there was no observable effect (Mooseker and Tilney, 1975). However CB did not interfere with the actin-filamin interaction in the absense of Tm-Tn (Wang and Singer, 1977; Davies, et al 1977) suggesting that ABP and filamin are not identical. It has been suggested that the two proteins are similar and cross reactive but not identical (Korn, 1978).

The binding of filamin to filamentous actin was further characterized (Wang and Singer, 1977) and it was shown that one filamin dimer existed per 8-12 actin subunits. Furthermore, filamin was shown to inhibit the actin-activated myosin Mg^{+2}-ATPase activity (Maruta and Korn, 1977; Davies, et al 1977), suggesting that when filamin is bound to actin *in vivo*, it is likely modulating a structural event rather than an energy requiring event. Further characterization of filamin has indicated that it is a protein which is phosphorylated in the presence of cAMP dependent protein kinase. However, there is no evidence yet that phosphorylation of filamin affects its interaction with actin or any other contractile protein (Wallach, et al 1978).

2.2.2.6 Spectrin

Another high molecular weight actin binding protein which has also been shown to be phosphorylated is spectrin. Spectrin has been classified as a dimeric protein in the pu-

rified state (Clarke, 1971) and immunological studies have indicated that spectrin is probably not found in cell types other than erythrocytes (Hiller and Weber, 1977). Spectrin was found to exist as a multimeric aggregate of two different proteins (220,000 and 200,000 daltons) in equal ratios. Characterization of spectrin at the cytoplasmic face of erythrocyte membranes began with the observation that it is similar to muscle myosin in terms of molecular weight, filamentous nature, and cross-reactivity between smooth muscle myosin antibodies and one or both of the proteins of the spectrin dimer. More significant to the present thesis was the finding that another protein associated with the spectrin was actin based on actin's ability to form 50-70 A filaments, to stimulate myosin ATPase activity, and to bind heavy meromyosin specifically (Sheetz, et al 1976). Characterization of the interaction between spectrin and actin followed, and evidence was presented to suggest that spectrin may act as a polmerizing factor for actin, as well as an anchoring site for erythrocyte actin (Puszkin, et al 1978). This idea was further substantiated when it was noticed that a complex isolated by binding to dihydrocytochalasin B from erythrocyte membranes containing actin, spectrin and other minor components, was able to induce actin polymerization. The rate of actin polymerization was dependent on the amount of complex present. It was also established that low concentrations of cytochalasins D or E and dihydrocytochala-

sin B further inhibited actin polymerization. The relative success of the various cytochalasins in inhibiting actin polymerization corresponded to their relative affinities for the complex and their potencies in inhibiting cell motility. It was then suggested that the cytochalasin binding complex functioned as a regulatory site for cell motility by controlling membrane attachment as well as formation of actin containing microfilaments in the cell (Lin and Lin, 1979). Recent studies on the effects of the spectrin complex on actin polymerization have indicated that this complex of actin and spectrin, may be important in the nucleation reaction (Brenner and Korn, 1980). Using partially purified spectrin which still contained a small amount of actin, an independent group also concluded that spectrin was involved in nucleation (Sato, et al 1979). However, the same researchers concluded that spectrin was not involved in cross-linking actin filaments which is in contrast to earlier results obtained by Brenner and Korn (1979). Brenner and Korn showed that although "unfractionated" extract of spectrin/actin complex induced G-Actin to polymerize, "column purified" spectrin tetramer or dimer did not. Conversely, these authors found that column purified spectrin tetramer cross-linked F-actin to form a highly viscous gel. Spectrin dimer which had been column purified, bound but did not cross-link F-actin. It was also shown that the state of spectrin phosphorylation did not affect its ability to cross-link actin

(Brenner and Korn, 1979; Anderson and Tyler, 1980). In another study, these findings were substantiated and it was suggested that since spectrin is likely present in the cytoskeletal network *in situ* as a tetramer which binds F-actin, it is unlikely that actin exists in its dimeric form in the erythrocyte membrane (Ungewickell, et al 1979). Liu and Palek (1980) have recently presented evidence to suggest that spectrin dimers and tetramers are in a reversible equilibrium in the membrane and that under physiological conditions, spectrin tetramers are favoured. To confirm this finding, it was also shown that if the dimeric state of spectrin is induced, the structural stability of the ectoplasmic layer of cytoplasm is disrupted. This suggested that spectrin in its tetrameric state offers the structural stability seen in erythocyte membranes.

2.2.2.7 Thyone Proteins

In *Thyone* sperm, high molecular weight proteins have been found to maintain actin in an aggregated state. The subacrosomal cup of *Thyone* sperm has been found to contain four major proteins including actin (Tilney, et al 1973). Two of the four proteins have a molecular weight of 250,000 daltons and have been implicated in maintaining the aggregated state of non-filamentous actin, since the dense gel-like cups dispersed when these high molecular weight proteins were removed (Tilney, 1976). Although these proteins are not pro-

moting the polymerization of actin, they may have some relationship to ABP and filamin.

2.2.2.8 Fibronectin

The relationship of the Ihyone sperm high molecular weight proteins to fibronectin has also not been fully established. Fibronectin is another high molecular weight protein (250,000 daltons) which is also know as FS (fibroblast surface) antigen, CSP (cell surface protein), LETS (large, external, transformation sensitive) protein, Gap a (galactoprotein a), zeta, and CIG (cold insoluabe globulin). The importance of fibronectin in relation to F-actin came from observations that a loss of fibronectin from the cell surface, induced by either viral transformations (Hynes 1973; Keski, et al 1979; Rsoslahti 1973) or by treatment with trypsin (Hynes 1976; Vaheri, et al 1976) coincided with the disorganization of actin containing stress fibers (Altenburg and Steiner, 1975; Edelman and Yahara 1976; Goldman, et al 1973; Goldman and Knipe 1972; Goldman, et al 1976; Pollack, et al 1975; Pollack and Rifkin 1975; Wang and Goldberg 1976). If fibronectin was added to transformed cells which had lost both fibronectin and actin stress fiber organization, the stress fibers reorganized (Ali, et al 1977; Yamada, et al 1976). It was recently suggested that fibronectin and actin fibers of the fibronexus (the association of fibronectin with actin across the plasma membrane) are

closely associated, with maximal separation of 8-22 nanometers. It is not known whether end-to-end (coaxial) or overlapping ends with binding, is responsible for the close apposition. Apart from this apparent role of fibronectin in maintaining the cytoskeleton, it has also been suggested that the fibronexus may serve as a site for cellular locomotion. When fibronectin was added to normal or tranformed cells, migratory activity was increased, along with increased actin organization as already mentioned. It may also be possible that the fibronectin is acting as a nucleating centre for actin bundle formation (Singer 1979).

2.2.2.9 Acanthamoeba castellanii gelactins

There have been a number of lower molecular weight proteins from Acanthamoeba castellanii as noted previously which have been shown to bind F-actin and lead to the formation of actin containing gels. Each of these lower molecular weight proteins (or gelactins) has been shown to be more effective than the macrophage ABP or smooth muscle filamin in gelling actin. The gelactins were also shown to inhibit the ability of F-actin to activate myosin Mg^{+2}-ATPase activity (Maruta and Korn 1977). There have been basically four gelactins isolated from A. castellanii namely: gelactin I (with 1 subunit of 23000 daltons), gelactin II (a dimeric prctein of 55000 daltons with a subunit of 28000 daltons), gelactin III (a dimeric protein with a subunit weight of 32000 daltons

and a total weight of 68000 daltons), and gelactin IV (total weight of 78000 daltons with a subunit of 38000 daltons). It is not known to what extent similar low molecular weight proteins are found in other systems such as macrophage or cultured HeLa cells.

2.2.2.10 Other proteins

Apart from the documented gelactins of A. castellanii, a number of other proteins have been shown to interact with F-actin by Laki and Muszbek (1974).

They demonstrated that F-actin was attached to fibrin and fibrinogen clots after the clot was separated from its liquor. In control experiments, actin did not bind to clots consisting of only fibrinogen. Furthermore, in experiments where:

1. clots of thrombin, fibrinogen and F-actin are formed
2. the thrombin is inactivated
3. the clots are dissolved in 6M urea (conditions where F-actin and fibrin depolymerize)
4. the solution is dialized to remove the urea
5. the clots are reformed

actin again bound to fibrin suggesting that the interaction of fibrin with actin did not depend on the presence of thrombin. To show that the interaction between actin and fibrin was specific, ovalbumin, which has a similar molecular weight to actin, was incubated with the clots containing

fibrin and no binding took place. Binding studies indicated that two actin molecules bound to one duplex fibrin molecule of molecular weight 330,000 daltons. The authors speculated that this binding of F-actin to fibrin but not to fibrinogen may be connected to the role of platelets in clot retraction since actin has been found in high concentration in these cells.

Another platelet protein involved in clot biochemistry is thrombin which is thought to interact with actin (Carlsson, et al 1979). In their studies it was shown that thrombin led to rapid stimulation of actin polymerization which corresponded temporally to the formation of pseudopods in platelets. However, it was noted that the changes in actin organization may also have been reflecting interaction with other components in this complex system.

Another protein which has been found to associate with actin is a polypeptide weighing between 52000 and 58000 daltons. This protein is left along with actin and a high molecular weight protein (250000 daltons) when tissue cultured cells are incubated with Triton-X-100. This detergent removes the outer membranes leaving the cytoskeleton exposed. Although the protein remaining appears to be associated with actin, the nature of the interaction has not been described. It has been suggested that the 52000-58000 dalton protein may be similar to the 100 A intermediate filament protein (Cooke 1976; Lazarides and Lindberg 1976; Starger and Gold-

man 1977). A protein of 52000-58000 daltons, called desmin, has been idenfified in a number of cell types particularily in muscle (Izant and Lazarides 1977; Lazarides and Lindberg 1976). In Limulus sperm, a protein of similar molecular weight, called scruin, has been identified together with α-actinin (Rosier, et al 1977; Tilney 1975). It is not known whether scruin and desmin are related or how any of these proteins of this molecular weight group are related to either the intermediate filament protein also with a molecular weight of 55000 daltons and/or the 52000-58000 dalton protein of the triton-X-100 cytoskeleton.

Tubulin (55000 daltons) is thought to be quite different from the previous proteins of similar molecular weight. However, it does appear to share the ability to interact with actin. In a recent paper, Griffith, et al (1978) showed that the mixtures of microtubules, microtubule associated proteins (MAPs), and actin have much higher viscosities than the sum of the individual components suggesting increased polymerization of actin and/or tubulin was occurring compared to the control with either actin or tubulin. Furthermore, the MAPs caused an increase in actin polymerization. If MAPs were absent from mixtures of actin and tubulin, the viscosities were close to the sum of the individual components. It was then suggested that there was an interaction between tubulin and actin which required the presence of the MAPs in the absence of ATP. Electron mi-

croscopy supported this contention since numerous close associations could be observed between the two proteins.

2.3 ENZYMES WHICH BIND F-ACTIN

Not all proteins associated with F-actin are structural proteins, and in fact, some of the structural proteins known to interact with actin, may be found to have their own enzyme activities in the future.

There is growing evidence to suggest that actin serves as a cytoplasmic compartment upon which many enzymes reside under various conditions. The idea of enzymes associated with a particular metabolic function residing together in a specific compartment is not new. The enzymes of the Krebs cycle were found to be located in the mitochondria, the enzymes of glycogen metabolism were found complexed to glygogen particles (Meyer, et al 1970), while the enzymes mediating the pentose-phosphate shunt have been found to be microsomal (Srere and Mosback 1974). For the purpose of this review, only the glycolytic enzymes thought to be associated with the newly proposed compartment 'F-actin' will be considered.

2.3.1 Aldolase

Some of the earliest work done in this area was performed by Arnold and Pette (1968) when they investigated the binding of pure rabbit muscle aldolase to F-actin, myosin, acto-

myosin, and the stroma protein of rabbit skeletal muscle. F-actin exhibited the greatest affinity and binding capacity for aldolase. Since aldolase has been documented to the greatest extent and studied in depth, it will serve as a typical example of glycolytic enzymes which bind actin.

2.3.1.1 Binding Characteristics

Arnold and Pette (1970) and Arnold, et al (1971) have demonstrated that by varying the pH, ionic strength, and the presence or absence of various substrates, the ability of aldolase to bind F-actin is changed and the kinetic parameters of free and bound aldolase are different. Not only was aldolse shown to bind F-actin, but, by using histochemical techniques, it was localized in the I-bands of skeletal muscle where actin is localized *in vivo* (Pette and Brandair 1962; Sigel and Pette 1969; Arnold, et al 1969). These histochemical findings suggested that the observed binding of aldolase to F-actin may not be an experimental artifact but may occur *in vivo*. Despite this evidence that aldolase does bind F-actin, it was noticed that binding only occurred at low ionic strength (<20mM KCl) and low pH (6.0-6.5) both of which are non-physiological. If the ionic strength or pH was increased above these values, there was a marked desorption of the enzyme from F-actin (Clarke and Masters 1972). However in a later report, Clarke and Masters (1973) suggested that the failure of aldolase present in myogen (a

preparation containing soluble proteins including all the glycolytic enzymes) to bind F-actin may have been due to the low protein concentrations used during the adsorption studies.

Aldolase could be induced to bind F-actin-Tm-Tn at low protein concentrations and physiological ionic strength and pH only if myosin was present (Clarke and Masters 1974). It was later conclusively demonstrated that aldolase would bind F-actin or F-actin-Tm-Tn without myosin under physiological ionic strength and pH provided the myogen concentration was high (about 50.0 mg/ml) (Clarke and Masters 1975). Although this protein concentration seems high, it should be noted that 50.0 mg/ml is lower than the proposed protein concentration of the I-band in skeletal muscle. Here it has been estimated that the myogen concentration within the interfilamentory sarcoplasm may exceed 200.0 mg/ml (Czok and Bucher 1960; Pette and Brandon 1962).

In a later study, the adsorption of aldolase to F-actin-Tm-Tn was examined in greater detail using electron microscopy (Morton, et al 1977). It was observed that, in the absense of troponin, small aggregates of aldolase and F-actin were formed. A lattice formation emerged in the presence of fully reconstituted filaments, and evidence was presented to suggest that the lattice was formed by an interaction between troponin and aldolase. This was supported by the observation that the lattice formation could occur with a min-

imum Tn subunit structure of Tn-T:Tn-I indicating that Tn-C and tropomyosin were not required for the aldolase binding. Further support for the idea that aldolase was binding Tn was provided by the lattice spacing of 38.7 nM which is consistent with X-ray diffraction studies of muscle fibers labeled with anti-Tn-C antibodies where the Tn repeats were found to be separated by 38.5 nM.

Walsch, et al (1980) have recently investigated the binding of aldolase to actin containing filaments by moving-boundary electrophoresis. It was found that there was a salt-dependent association between Tm alone, Tn alone, and a complex of Tm-Tn with aldolase. The enzyme had the greatest affinity for the Tm-Tn complex. Furthermore, the stoichiometry of the binding of aldolase to actin was markedly influenced by the presence of Tm and Tn. With F-actin, only, there was one tetrameric aldolase molecule per 14 monomeric actin molecules, however, if F-actin-Tm was the adsorbent, the stoichiometry was 2:14. This stoichiometry was further increased to 4:14 if F-actin-Tm-Tn was present and the binding constant for aldolase association was decreased by an order of magnitude. If 0.1mM $CaCl_2$ was included, the stoichiometry decreased to 3:14 with F-actin-Tm-Tn.

The characteristics of the binding stoichiometries was further analyzed in an accompanying paper again using electron microscopy studies (Stewart, et al 1980). Their data demonstrated that paracrystals formed between aldolase and

F-actin or F-actin-Tm contained a single light transverse band every 38 nm due to the cross-linking of the actin filaments by aldolase. However if actin was adsorbed to F-actin-Tm-Tn, the paracrystals had 2 transverse bands every 38 nm. The major band was considered to represent aldolase binding to troponin and the minor band was thought to be aldolase cross-linking the filaments. When the Ca^{+2} concentration was low, the intensity of the minor band was greatest. Based on these facts, plus confirmed knowledge of the stoichiometrics of actin:Tm:Tn and their structure relative to each other, a model was proposed to explain their observations. The 1:14 stoichiometry of aldolase to F-actin was explained by the assumption that aldolase can only bind the filaments where it can bind two G-actin subunits simultaneously. Since actin is a helix, it would be expected that this would occur twice in the helix length (since it is double stranded) or every 38 nm, as was observed. This length of 38 nm corresponds to 7 actin monomers, however since each aldolase binds two actin monomers, the stoichiometry would be 1:14 (aldolase to actin) instead of 1:7. The doubling of the stoichiometry to 2:14 in the presence of F-actin-Tm was explained by suggesting that aldolase contains a Tm binding site. Since the pitch of the Tm helical path is the same as that of actin, it was suggested that same number of cross-links would form as for G-actin alone. However, the two helices are not in phase with the actin helices and it was

suggested that the binding site for the two types of cross-link would occur at slightly varying axial positions along the thin filament. This remains to be confirmed. The increase in stoichiometry of aldolase binding in the presence of Tn and Ca^{+2} to 3:14 was suggested to be due to a Tn-binding site on aldolase. When Ca^{+2} was absent, the increased stoichiometry (4:14) was thought to be due to one aldolase binding actin and since there are two Tn molecules per repeat and aldolase binds Tn, a further addition of two more aldolase was included. The fourth aldolase was reported to be due to the binding of this enzyme to Tm. In the presence of Ca^{+2}, Tm is thought to move deeper into the actin groove and in this position, it is thought to be ineffective in binding aldolase. Only when Tn is present, can Ca^{+2} confer such a change in the position of Tm. It is thought that the movement of Tm inhibits its ability to bind aldolase because of stereochemical hinderance in the association between Tm-aldolase and actin induced by Ca^{+2}. This view has been recently supported by the finding by Walsh, et al (1980) who showed that the binding of aldolase to Tn-Tm without actin did not show Ca^{+2} sensitivity.

2.3.1.2 Physiological significance

Although it has been demonstrated that various components of the actin filaments bind aldolase, the question remains whether or not there is any physiological significance to

such a phenonena. It has been proposed that a possible physiological function in adsorption is that the binding of enzymes, such as aldolase, to particulate structures, such as the thin filaments, may be establishing a means of regulating kinetic activity. In fact, Arnold and Pette (1970) have demonstrated an alteration of aldolase kinetic parameters upon binding to F-actin. Walsh, et al (1977) illustrated that aldolase binding to F-actin-Tm was relatively similar to aldolase binding F-actin. However, upon binding F-actin-Tm-Tn, major modifications in the kinetic parameters of aldolase and an additional Ca^{+2}-sensitivity were detected. The Km was increased by 8 times and 12 times when compared to the soluble enzymes in the presence of F-actin and F-actin-Tm while the V(max) was altered only slightly. However, in the presence of F-actin-Tm-Tn and 0.2mM EGTA, the Km for aldolase binding was increased by 100 times and the V(max) by 4 times. If 0.1mM $CaCl_2$ was incorporated instead of EGTA, the Km was only increased by 20 times and the V(max) by 2 times. It was suggested that the Ca^{+2} may be regulating the activity of this glycolytic or energy producing enzyme, as well as triggering contraction. If the inhibition of aldolase was partially released by Ca^{+2} when aldolase activity was needed most (as was seen by the decreased Km in the presence of Ca^{+2} as compared to EGTA), some coordination between energy production and contraction may be taking place. Further support for the regulation of

energy production in tune with contraction by adsorbtion of aldolase originated from the observation that, at high concentrations (>30 um) of the enzyme's substrate (as occurs during contraction and rapid glycolysis), greater activity of the bound enzyme was noted than would be expected of the soluble enzyme at a similar substrate concentration.

Apart from regulating the activity of the enzyme, Clarke and Masters (1972,1973a) suggested that preferential adsorption of different isozymes of aldolase may differentiate between the functional roles of these proteins. In particular, aldolase A4 bound most firmly to a particulate fraction in brain followed by A3C, A2C2, AC3, C4. Previous to the discovery of a differential binding of the isozymes, no separate role could be attributed to the five proteins despite extensive analsysis of their catalytic activities. Knull, et al (1973) has shown that another glycolytic enzyme, hexokinase, is partitioned between soluble and particulate fractions in this tissue.

Dedman, et al (1975) also suggested that the binding of aldolase by actin increases its susceptibility to proteolytic attack. This became of physiological significance when it was noted that its substrate increased its binding to F-actin-Tm-Tn (Clarke and Masters 1975). During work, the substrate fructose 1,6-diphosphate increases due to glycolysis, which may lead to increased binding of aldolase to actin thereby rendering it more susceptible to proteolytic at-

tack. Based on this suggestion, it was predicted that in muscles with constant glycolytic activity (e.g. slow twitch oxidative fibers of heart and soleus), the degradation of aldolase would be enhanced due to its increased binding to actin in the presence of increased concentrations of glycolytic intermediates, such as FDP, when compared to muscles which had occassional spurts of glycolytic activity such as the fast twitch glycolytic fibers of psoas muscle. In fact, Goldberg (1967) and Dolken and Pette (1974) have suggested that the protein degradation rates are faster in the slow twitch fibers than in the fast twitch fibers. Although increased degradation may be taking place in vivo, it is difficult to understand what relationship this increased degradation rate has to providing energy for contraction unless there is also an increase in the rate of synthesis of the enzyme.

Further support for the idea that aldolase binding to actin is of physiological significance, has been extracted from data obtained by electrically stimulating muscles. Starlinger (1967) illustrated that aldolase adsorption increased with electrical stimulation of muscles and adsorption decreased with time after stimulation of the intact animal. The observation that alterations in the physiological status of the animal induced changes in the aldolase binding suggested that the phemomena may be of some significance. Clarke, et al (1980) have recently shown that in particulate

homogenates of electrically stimulated bovine psoas muscle, there was an increased glycolytic activity as well as an increased binding of aldolase to the particulate fraction. Calculations based on the extent of enzyme binding suggested that significant amounts of aldolase were associated with actin. It was therefore suggested that kinetic parameters based on enzymes in the soluble state may not be relevant to the *in vivo* situation, particularily during increased glycolysis.

It should be noted that aldolase is not the only enzyme which binds to actin during electrical stimulation. Glyceraldehyde-3-phosphate dehydogenase was shown to exhibit increased adsorption upon electrical stimulation. There was also significant binding of phosphofructokinase and pyruvate kinase to the particulate fraction. In fact, although the present review emphasized the characteristics of aldolase adsorption to actin, many glycogenolytic and glycolytic enzymes are found localized in thin filament regions. By using histochemical techniques, Pette and Brandau (1962), Sigel and Pette (1969) and Arnold, et al (1969) showed that phosphorylase, phosphoglucomutase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, aldolase and lactate dehydrogenase were located in the I-bands of skeletal muscle where actin is located. At this time it would appear that the glycogenalytic enzymes do not associate with actin *in vitro* and that only the glycolytic enzymes

bind directly to actin (Arnold, et al 1971). It would be ideal if the localization of many of the enzymes associated with energy production were at a site in the sarcoplasm where they could be most needed, i.e. next to the contractile fibers themselves.

Although it has been established that various glycolytic enzymes do bind to F-actin, there has been no in depth evaluation of their effects on actin polymerization.

2.4 Ca^{+2} REGULATION OF ACTIN GELATION

Although the intracellular microfilament system has been implicated in many biological processes, the mechanisms involved in controlling the organization of this cytoskeleton are poorly understood. However, since Ca^{+2} was found to affect the ability of actin to activate myosin ATPase activity and therefore contraction in muscle, it was questioned whether Ca^{+2} was also regulating the various functions of actin in non-muscle cells. As illustrated previously, F-actin has been shown to cross-link in the presence of certain proteins during gelation. The so formed gel has been shown to contract and researchers have considered this "non-muscle contraction" to be a reflection of contraction in muscle only on a microscale. The gelation process has been observed in extacts of many non-muscle cells such as rabbit pulmonary macrophages (Hartwig and Stossel, 1976; Stossel and Hartwig, 1979), HeLa cells (Weihing 1976; Weihing 1977),

human leucocytes from patients with chronic myelogenous leukemia (Boyer and Stossel 1976), platlets (Lucas, et al 1976), Acanthamoeba (Pollard 1976a; Pollard 1976b), Amoeba proteus (Taylor, et al 1976), and Dictyostelium discoideum (Condeelis and Taylor, 1977; Taylor, et al 1977). However the involvement of Ca^{+2} in the process of gelation was first implied when Kane (1975,1976) noticed that actin filaments formed gels when extracts of sea urchins were warmed in the presence of EGTA as well as ATP and KCl.

Mimura and Aano (1978) have demonstrated that Ca^{+2} could reversibly inhibit gelation with micromolar concentrations. They also made the interesting observation that this type of regulation may be involved in virus induced cell fusion. To support this contention, they recalled the data reported by Okanda and Murayama (1966) which showed that extracellular Ca^{+2} was required for maximum fusion of Ehrlich tumor cells induced by Sendai virus. Mimura further investigated Ca^{+2} regulation of these cells and found that the "intracellular" Ca^{+2} concentration seemed to be higher in cells resulting from virus induced fusion than in normal cells and that less Ca^{+2} was required to inhibit gelation of extracts from fused cells. These results seemed to imply that, in virally treated cells, the increased intracellular Ca^{+2} concentration and the decreased amount of Ca^{+2} required to inhibit gelation, ensured that actin would not gel. It was then suggested that Ca^{+2} may be controlling the intracellular organization of microfilaments during the fusion process.

The question of how the gelation process was regulated by free Ca^{+2} was considered in more detail (Ishiura, et al 1979). It was found, using Ehrlich Ascites tumor cells, that 0.06uM Ca^{+2} caused a 50% inhibition of gelation, and complete inhibition was achieved at 0.5 uM Ca^{+2}. Furthermore, it was shown that the inhibition could be reversed using excess EGTA. When gels formed with EGTA were overlaid with $CaCl_2$, 50% of the gel liquified (a process called solation) at about 0.6uM Ca^{+2}. The fact that concentrations of Ca^{+2} between 10^{-8} and 10^{-6}M controlled gelation, is physiologically significant since the concentration of Ca^{+2} in the cytosol of animal cells is in the range of 10^{-8}M to 10^{-5}M (Kretsinger 1976). Ca^{+2} had been implicated as being important in cell movement when Ridgway, et al (1976) illustrated that cyclic changes in ionized intracellular Ca^{+2} occurred in synchrony with streaming of Physarum polycephalum, a phenomena which depends on the aggregation state of actin. Goshina, et al (1978) had also found a similar phenomena in a mammalian system where it was shown that micromolar levels of free Ca^{+2} controlled cell shape and rhythmical beating of cultured mouse cardiac cells, events also thought to depend on actin's involvement in contraction. These reports suggested that fluctuating Ca^{+2} levels were regulating the activitsy of actin by controlling it state of gelation. Whether Ca^{+2} was directly controlling the aggregation state of actin or whether other components in the gel were sensi-

tive to Ca^{+2}, was first considered by Ishiura, et al (1979). Cobbold (1980) has recently shown by using calibrated aequorin measurements that changes in the pattern of streaming are not accompanied by changes in the aequorin signal for the amoeba Chaos carolinense. Although the cytoplasmic free Ca^{+2} was at a constant level, Ca^{+2} could still be influencing movement. The mechanism for this influence could be that the sensitivity of the cytoplasm towards Ca^{+2} could change via an alteration of the sensitivity of the contractile proteins to the cation.

2.4.1 Actinogelin

Mimura and Asano (1979), who had discovered the inhibition of gelactin by micromolar concentrations of Ca^{+2}, later found a Ca^{+2} sensitive protein factor in Erhlich Asites tumor cells (ETC). The protein isolated was found to have a molecular weight of about 115000 daltons as judged by SDS-polyacrylamide gel electrophoresis and was named actinogelin to distinquish it from other actin binding proteins. The content of actinogelin in crude extracts was estimated to be greater than 0.3%. This factor produced gels on mixing with rabbit skeletal muscle actin provided 1mM EGTA was present, and further characterization of the gel formed in the presence of actinogelin revealed that a molar ratio of approximately 1:100 actinogelin (calculated as a dimer) to actin, could produce detectable gelation. The molecular ratio of

actin to actinogelin at maximum binding was extimated to be 10:12. In terms of Ca^{+2} regulation, less actinogelin can be found bound to F-actin in the presence of Ca^{+2} as compared with controls in EGTA. At the molecular level, it is not known whether Ca^{+2} may be changing the quaternary structure of this Ca^{+2} sensitive protein in order to decrease gelation.

It is interesting to note that ETC's also contain another protein which cross-links actin filaments called filamin. Unlike actinogelin, filamin induces gelation in a Ca^{+2} insensitive manner, and therefore it seems as though two different gelation systems exist in ETC. Such bimodal regulation of gelation may exist in other cells where both of these proteins are thought to exist, such as in extracts of: leucocytes (Stossel and Boyer 1976), HeLa cells (Weihing 1977), and in amphibian cells (Clark and Merrian 1978).

2.4.2 Gelsolin

About the same time that Mimura, et al (1979) released their paper on actinogelin, Lin, et al (1979) independently reported the discovery of another Ca^{+2} dependant regulatory protein from rabbit macrophages which they termed gelsolin. Previous to their discovery, it had been reported that the activities in macrophages and many eucaryotic cells, namely locomotion, secretion and endocytosis, are thought to take place in the peripheral cytoplasm. It has been observed

that this area, which contains many actin filaments, appears to fluctuate between conditions of gelation and solation during the various activities. Once again, cell extracts were used to study the phenomena of gelation/solation transformation. In these extracts, actin filaments were noted to be arranged in tangled arrays and these cross-linked arrays were sensitive to Ca^{+2}. ABP is responsible for the cross-linking, however it was found to be insensitive to Ca^{+2} in terms of its ability to induce gelation. Further investigation led Lin, et al to discover that gelsolin was responsible for the Ca^{+2} sensitivity in the gelled extracts. The molecular weight of gelsolin was estimated to be about 160000 daltons based on the elution patterns of globular proteins with known molecular weights from gel filtration columns. SDS-polyacrylamide gel electrophoresis suggested a molecular weight of 91000 daltons and therefore the authors suggested that the protein exists as a dimer in its native form. Unlike actiogelin, gelsolin alone will not induce actin gelation. However, actin gelation by macrophage ABP became sensitive to Ca^{+2} when gelsolin was included. The inhibiting effect of gelsolin on gelation in the presence of Ca^{+2} could be largely reversed by EGTA. Furthermore, the concentrations of free Ca^{+2} used to inhibit gelation of actin are similar to those required to inhibit gelation of cytoplasmic extracts of macrophages and therefore are likely within the limits observed for living cells.

Studies were also performed to determine the site of action of gelsolin and it was suggested that gelsolin exerted its effect by binding to actin in the presence of Ca^{+2} since a large amount of gelsolin was found in pellets of F-actin cross-linked with filamin. Furthermore, the possibility that gelsolin was interacting with filamin was eliminated. Lin, et al proposed that gelsolin divided actin filaments between cross-linking points leading to an increase in the number of filaments resulting in a disruption of the cross-linked network. This mechanism for disruption was based on an established theory for polymer gels which predicted that small deviations in the cross-link to polymer ratio near a certain point can abruptly alter "gel-sol" transformations (Flory, 1941). Actin-ABP networks behave in a manner which agrees with this theory (Brotschi, et al 1978). According to the model, if gelsolin divided the actin in filaments while the number of cross-links remained the same, the net increase in the number of filaments occurring when gelsolin bound Ca^{+2} would decrease the cross-link:polymer ratio leading to a dissolution of the gel. Furthermore, the small amount of gelsolin required to inhibit gelation of actin is consistent with the idea that gelsolin can dissolve actin gels by making a few lesions in the actin filaments. It was concluded that gelsolin is a physiological calcium dependent regulator of macrophage cytoskeletal structure (Lin, et al 1979).

Stendahl and Stossel (1980) have recently implicated gelsolin not only in cytoplasmic structure, but also in cell movement in a non-muscle systems. Actin in some non-muscle cell cortices exists as a three dimensional isotropic meshwork (Wessels, et al 1971) and myosin distributed throughtout this network, *in vitro*, can generate a contraction (superpreciptation) in the presence of $MgCl_2$ and ATP. The only orientation of force generated in a such a system is centripetal, or towards the centre of the network. However, it was proposed that directionality in movement could be obtained if there were differences in the efficiency of contraction of the lattice structure relative to different areas. Wessels, et al (1971) described the formation of an actin meshwork formed in the presence of ABP and demonstrated that this meshwork enhances the efficiency of contraction when myosin is also present. Furthermore, it was shown that Ca^{+2}-activated gelsolin reversed this enhancement and these changes lead to directional movement. For example, ABP reduced by four times the concentration of mysoin required for half-maximal contraction. In the presence of Ca^{+2} and gelsolin, the enhanced effect on contraction was abolished. However, if Ca^{+2} and gelsolin were added to only one side of the gel, the remainder of the gel moved away from this side and the time necessary for the gels to contract was halved. The model proposed that the mechanism of movement was based on ABP serving to cross-link actin fila-

ments and thereby adding them to the contracting mass. By recruiting the filaments in this manner, amplification of the myosin ATPase activity was achieved, i.e. less myosin per actin filaments would be required if the actin filaments are connected to each other. When the network was disrupted elsewhere by gelsolin and $CaCl_2$, the opposing forces in the opposite direction led to movement in a direction away from the solating gel, i.e. the regions which were more highly cross-linked had a greater efficency of contraction relative to solating areas, and therefore, tended to move away from areas of fewer cross bridges. Only that part of the cell attached to these areas would move accordingly.

This was one of two recent proposals set forth to explain how actin and myosin could interact in a non-muscle system to generate force. However, this mechanism has not been established, and it is generally believed that the processes underlying the regulation of contraction in non-muscle systems to bring about the varied response of living organisms is likely to be complex. It is also believed however, that fluctuations in the concentration of intracellular Ca^{+2} are the basic regulators of contractile activity in non-muscle systems as they are in muscle systems.

Calmoldulin via a concerted interaction with calcium, is involved in a second more widely confirmed mechanism for controlling non-muscle contractile activity. The regulation of contractile activity by CM has already been demonstrated

in smooth muscle (Chacko, et al 1977; Small and Sobieszek 1977; Gorecko, et al 1976), and similar mechanisms for non-muscle movement have been proposed in tissues such as platelets (Adelstein and Conti, 1975), and baby hamster kidney cells (Yerna, et al 1979). In these systems, the mechanisms of activation of contraction is via a CM and Ca^{+2} activated myosin light chain kinase. This enzyme was found to phosphorylate the 20000 dalton light chains of myosin and this activity was enhanced in the presence of CM and Ca^{+2}. With an enhanced phosphorylation, actin was able to increase the activation of myosin Mg^{+2}-ATPase by at least 5-8 times.

Although a regulation resulting from the interaction of CM with actin rather than myosin was sought in the present project, it should be noted that CM is by no means restricted to the regulation of myosin light chain kinase. In fact, CM has been implicated in many different cellular activities which previously were discovered to be regulated by Ca^{+2} (Wang and Waisman, 1979). In relation to the present thesis, it is interesting to note that Ca^{+2} has been found to regulate the interaction of actin with other actin binding proteins, and the question remains as to whether CM may also be regulating these phenomena.

2.5 CA^{+2} REGULATION OF CELLULAR ACTIVITIES

2.5.1 Calmodulin

The various cellular activities which have already been found to be regulated by calmodulin include cyclic nucleotide and Ca^{+2} metabolism, muscle contraction, secretory processes, microtubule assembly, mitotic movements, and glycogen metabolism. In fact, the name calmodulin was coined by Cheung (1978) to indicate its ability to modulate the effects of Ca^{+2} in these diverse cellular processes. Furthermore, calmodulin was shown to bind Ca^{+2} by Teo and Wang (1973).

2.5.1.1 Ca^{+2} Binding and Conformation

The interaction between Ca^{+2} and CM has been characterized in several laboratories, particularily the binding of Ca^{+2} by CM and the conformational changes induced in CM by calcium. There appears tc be some controversy over the number of binding sites for Ca^{+2} and the dissociation constants. It has been suggesed that this may be due to variations in the concentrations of Mg^{+2} in the buffers used, changes in ionic stregth of buffers, trace contamination of Ca^{+2} in CM samples, different means of determining Ca^{+2} or protein contration, or denaturation of CM during binding studies (Wang and Waisman 1979). Despite the discrepencies reported, there are some generalizations which can be made about the Ca^{+2} binding characteristics. It has been shown

that CM binds a maximum of four moles of Ca^{+2} per mole of protein and the dissociation constants at high ionic strength (>40mM) are in the micromolar range. Millimolar Mg^{+2} concentrations have no effect on the Ca^{+2} binding, however at low ionic strength, Ca^{+2} and Mg^{+2} may compete for binding sites. Different classes of binding sites have been documented, but no positive cooperativity has been shown for Ca^{+2} binding.

It has been generally agreed that Ca^{+2} causes a conformational change in CM by increasing its helical content. From a general point of veiw, Ca^{+2} is thought to transform CM from a less ordered entity to one that is more ordered, symmetrical, compact and stable (Wang and Waisman, 1979).

Wolff, et al (1977) has shown that the microenvironment of aromatic residues upon the binding of Ca^{+2} by CM had UV absorption peaks at 258, 262, 269 and 280 nm. These results suggested that the tryosine and phenylalanine microenvironments were affected by Ca^{+2}. Klee (1977) made the same conclusion based on the difference UV-absorption spectrum of the protein, however, Liu and Cheung (1976) maintained that Ca^{+2} had no effect on the UV absorption spectrum. Less controversial were the chemical modification studies and spectroscopic titrations used to examine the microenvironment of tyrosine residues 99 and 138 of bovine brain calmolulin (Richmond and Klee, 1978). Their general conclusion was that tyrosine 138 is in an unusual environment based on its

varying dependence for Ca^{+2} in order to be modified and the unusually high pK values of nitrotyrosine 138. Furthermore, the pK value of nitrotyrosine 138 could be altered depending on whether or not Ca^{+2} was present again suggesting that this amino acid was in an unusual environment.

Walsh and Stevens (1977) further studied the Ca^{+2} binding environment using chemical modification and indicated that, in the presence of Ca^{+2}, the rates of carbethoxylation of the sole histidine, and nitrification of the tryosine were increased. Conversely, in the presence of Ca^{+2}, it was shown that the rates of inactivation of calmodulin by group specific reagents was decreased. Also, by comparing cyanogen bromide peptide maps of these modified proteins, it was shown that different methionines were modified in the presence and absence of Ca^{+2}. The methionines near the centre of CM were modified only in the presence of Ca^{+2} suggesting that it is this area of the molecule which is important in the binding of Ca^{+2} (Walsh, et al 1978).

It has been proposed by Klee (1977) that CM has two high and two low affinity binding sites for Ca^{+2}. Utilizing both UV difference spectroscopy and the UV circular dichroism spectrum, it was shown that the binding of two Ca^{+2}'s by CM brought about more that 80% of CM's conformational change. Given that CM does bind Ca^{+2} and that this binding induces a conformational change in the molecule, the question remains how this Ca^{+2} binding protein activates the many proteins to which it is associated.

2.5.1.2 Mechanism of Enzyme Activation

Wang, et al (1975) have proposed a general mechanism for the activation of enzymes by Ca^{+2} and CM based on the experimental results obtained from several laboratories. The first step is thought to be the binding of Ca^{+2} to CM with the subsequent conversion of CM from its inactive to active conformation. The activated CM is then thought to associate with the enzyme thereby converting it from an inactive to an active protein as illustrated in the following scheme:

$$Ca^{+2} + \underset{\text{(inactive)}}{CM} \longleftrightarrow \underset{\text{(inactive)}}{Ca^{+2}\text{-}CM} \longleftrightarrow \underset{\text{(active)}}{Ca^{+2}\text{-}CM^{*}} \qquad (1)$$

$$\underset{\text{(inactive)}}{Ca^{+2}\text{-}CM^{*}} + E \longleftrightarrow Ca^{+2}\text{-}CM^{*}\text{-}E \longleftrightarrow \underset{\text{(active)}}{Ca^{+2}\text{-}CM\text{-}E^{*}} \qquad (2)$$

2.5.1.3 PDE

The first aspect of reaction (1) involves the binding of Ca^{+2} to CM. Teo and Wang (1973) were the first to illustrate this using gel filtration. It was shown that $^{45}Ca^{+2}$ eluted from the gel column at a position which corresponded to the CM activity peak, thereby indicating that Ca^{+2} bound to CM. Furthermore, to confirm this observation, Wang, et al (1975) showed that CM dependent phosphodiesterase (PDE) was incapable of high affinity binding of Ca^{+2} as was shown for CM. This illustrated that it was the CM which was binding Ca^{+2} and not the various CM-regulated enzymes such as phosphodiesterase. The second aspect of reaction (1) in-

volves the conformational change of CM upon binding Ca^{+2} as previously outlined. This has been implicated by ultraviolet absorption, circular dichroism, spectroscopic titration and reactivity of amino acid residues. Other techniques such as resistance to proteolysis (Ho, et al 1975; Liu and Cheung, 1976), fluorescence emmission (Dedman, et al 1977; Wang, et al 1975; Drabikowski, et al 1977), optical rotatory dispersion (Liu and Cheung, 1976), sedimentation constant and Stokes radius (Kuo and Coffee 1976) have indicated similar findings.

The fact that Ca^{+2} is required for the binding of the Ca^{+2}-CM complex to the enzyme(s) as well as for activating CM has been shown by Teshima and Kukiuchi (1974), and Lin, et al (1975). The enzyme used in their studies was the CM-dependent PDE. It was shown that in the presence of EGTA, CM and PDE eluted at different positions from a sephadex G-200 column whereas in the presence of Ca^{+2}, the two proteins were eluted together. These results suggested that Ca^{+2} was required for the association of CM with PDE. Although the dependence on Ca^{+2} for the association of CM and PDE was established, the stoichimetry of the interaction has not been determined.

Indirect evidence has suggested that the binding of CM-Ca^{+2} to PDE results in a conformational change to PDE as illustrated in sequence (2). This is based on thermal studies where it has been shown that, at $5^{0}C$, the stability of PDE

was decreased in the presence of CM and Ca^{+2} (Ho, et al 1977; Wolff and Brostrom, 1976; Wang, et al 1975; Liu and Cheung, 1976). Also of importance is the fact that certain phospholipids have been found to activate PDE (Hidaka, et al 1978; Wolff and Brostrom, 1976; Pichard and Cheung, 1977). Again in the presence of phospholipid activators, a decrease in the thermal stability of the enzyme was noted. However Ho, et al (1977) maintains that the thermal stability is increased in the presence of the activators CM and Ca^{+2}. In any case, some change in the conformation may be inferred by the "altered" heat resistance.

The characteristics of PDE activation by Ca^{+2} and CM have been studied in great detail. It has been found that at saturating levels of Ca^{+2}, 10 ng/ml of pure CM leads to a 50% activation of PDE (Teo and Wang, 1973). On the other hand, the concentration of Ca^{+2} required for activation depends on the level of CM in the assay. When CM is saturating, 2.3 uM Ca^{+2} leads to a 50% activation of the enzyme. Values varying between 1 to 8 uM have been obtained from other investigators. Since the Ca^{+2} concentrations in the cytosol varies between 10^{-7} and 10^{-5} (Berridge 1975), the Ca^{+2} requirements for CM are physiological suggesting that this mechanism operates <u>in vivo</u>. Other metals such as Sr^{+2}, Mn^{+2}, Ba^{+2}, and Co^{+2} can replace Ca^{+2} in activating the enzyme, but they are an crder of magnitude less efficient (Kakiuchi, et al 1973; Lin, et al 1974).

In the presence of EGTA, the activation of PDE by CM can be reversed (Lin, et al 1974; Brostrom and Wolff 1976). Other inhibitors of PDE include pharmacological antiphyschotic drugs (Weiss, et al 1974). It was found that more that 10 antipsychotic drugs inhibited the CM activated PDE activity and that the potencies of their inhibitory activity paralleled their effectiveness as antipsychotic agents (Levin and Weiss, 1976, 1977; Weiss and Levin, 1978). It is thought that the inhibition is based on a Ca^{+2} dependent, EGTA reversible association of CM with the drug which prevents CM from activating PDE.

PDE was the first enzyme discovered to be regulated by Ca^{+2} bound to CM. In fact, may forms of PDE exist in mammalian tissues, but the enzyme considered in the preceding paragraphs was the CM dependent enzyme which catalyzes the hydrolysis of both cAMP and cGMP. The V(max) for cAMP hydrolysis is severalfold higher that that for cGMP, however, the affinity of PDE for cGMP is greather than for cAMP. It should be noted that CM-independent forms of PDE exist and the enzyme has often been classified based on this dependence. It has also been classified based on its varying affinities for cAMP and cGMP and its distribution within the cell, i.e. cytosolic or membrane bound.

2.5.1.4 Adenlyate Cyclase

Since the discovery of CM-dependent PDE, many other enzymes have been discovered which are regulated through the modulatory activity of CM in the presence or absence of Ca^{+2}. Adenylate cyclase is one such enzyme. Bradham, et al (1969) originally demonstrated the dependence on Ca^{+2} for its activity and EGTA for its inactivity. Brostrom, et al (1975) and Cheung, et al (1975) later illustrated that the Ca^{+2} activation of the enzyme was mediated by CM. Further study has indicated that the mechanism of enzyme activation is similar to that reported for PDE (Lynch, et al 1976). The mechanism of CM activation of adenylate cyclase has also been suggested to be biphasic. Evidence for this proposal has been based on the observations of Brostrom, et al (1977) that adenylate cyclase, from particulate fractions of rat cerebral cortex, displays a biphasic dependence on both Ca^{+2} and Mg^{+2}. For both of these cations, low concentrations activate the enzyme and high concentrations inhibit it. It was suggested that the binding of Ca^{+2} to the high and low affinity sites of CM, led to the enzyme's activation and inhibition. The inhibition from Mg^{+2} was suggested to be based on its binding to CM's high affinity Ca^{+2} binding sites. Further evidence for the suggestion that CM and Ca^{+2} lead to the activation as well as the inhibition of adenylate cyclase by Ca^{+2}, was based on the observations that at low concentrations of Ca^{+2} and CM, the enzyme is activated, but at low Ca^{+2} concentrations and high CM concentrations,

the enzyme is inhibited. When high Ca^{+2} concentrations were used, only inhibitory activity of CM was expressed towards adenylate cyclase (Brostrom, et al 1977).

2.5.1.5 Myosin Light Chain Kinase

Myosin light chain kinase is another enzyme whose activity is regulated by CM. This enzyme is responsible for the phosphorylation of the 20000 daltons light chains of myosin in the presence of Ca^{+2} (Pries, et al 1974). There is much evidence supporting the idea that the phosphorylation of these myosin light chains controls the actomyosin ATPase and therefore the contractile apparatus in non-muscle cells and in smooth muscle. Adelstein, et al (1977), Dabrowska, et al (1977) and Yazawa and Yagi (1977) illustrated that two protein components were required for the activity of the enzyme in smooth muscle. The small molecular weight component was shown to be CM based on its molecular characteristics, its ability to activate PDE and its ability to reconstitute myosin light chain kinase activity (Dabrowska, et al 1978; Yagi, et al 1978). The fact that CM stimulates both mysoin phosphorylation and actomyosin ATPase activity implies that it is a regulator of Ca^{+2} function in smooth muscle contraction and in various non-muscle contractile activites. One non-muscle contractile activity which is thought to depend on the CM activation of myosin light chain kinase is neurotransmitter release. In brain, CM - Ca^{+2} complex has been

shown to activate a myosin light chain kinase and at the same time, regulate neurotransmitter release (Babitch, et al 1979). However, exactly how these two events are coupled *in vivo* has not been conclusively demonstrated.

2.5.1.6 Phosphorylase Kinase

Calmodulin has also been shown to activate two enzymes which are associated with glycogen metabolism. The first enzyme to be considered is phosphorylase kinase which is an enzyme forming part of the cascade leading to glycogen breakdown. Although the conversion of phosphorylase kinase to an active enzyme is dependent on the cAMP dependent protein kinase, and therefore is subject to control mechanisms affecting the level of cAMP, only Ca^{+2} regulation of the enzyme will be considered here.

Recent data has suggested that in 0.1M NaCl, phosphorylase kinase binds 8 moles of Ca^{+2} per mole of the enzyme with a dissociation constant of 5.5×10^{-7}M. Two classes of binding sites, high and low affinity sites, have been described for the enzyme in the presence of 20mM Mg^{+2}. Under this condition, 8 moles of Ca^{+2} bound per mole of enzyme to high affinity sites, with a dissociation constant of 2×10^{-6}, and four moles of Ca^{+2} per mole of enzyme bound to the low affinity sites with a dissociation constant of 3.5×10^{-5}M (Kilimann and Heilmeyer 1977).

Originally, phosphorylase kinase was proposed to have a subunit structure of $\alpha\beta\gamma_4$ with molecular weights 145000, 128000, and 45000 daltons respectively. More recently, it has been proposed that phosphorylase kinase contains a fourth subunit (Δ) in near stoichiometry with the other subunits. In fact, this fourth subunit has been identified as calmodulin (Cohen, et al 1978) based on its amino acid composition, UV spectra, electrophoretic mobility, and its ability to activate PDE and myosin light chain kinase.

This tight association of CM with phosphorylase kinase makes the activation of the enzyme unique among CM activated proteins. With other enzymes, CM forms a Ca^{+2} dependent association, whereas with phosphorylase kinase, the CM is permanently tightly bound to it. Although CM is tightly bound to the enzyme additional activation of phosphorylase kinase can be brought about by "exogenous" CM in the presence of Ca^{+2} (Waisman 1979). The interaction between phosphoylase kinase and exogenous CM was shown to be reversible since CM bound sepharose 4B, released the enzyme in the presence of EGTA but retained it in the presence of Ca^{+2} (Waisman, 1979).

2.5.1.7 Glycogen Synthase

Glycogen synthase is another enzyme related to glycogen metabolism whose activity was shown to be affected by CM. CM was found to stimulate the rate of phosphorylation of

this enzyme, thereby inactivating it (Strivastas, et al 1979). However, the protein kinase responsible for the phosphorylation has not been identified to date. It is interesting to note that CM serves to activate glycogen breakdown by increasing the activity of a glyogenolytic enzyme (phosphorylase kinase) as well as decreasing the activity of a synthetic enzyme, glycogen synthase. In this manner, CM serves to regulate glycogen metabolism in a coordinated fashion.

2.5.1.8 Membrane Phosphorlyation

Last to be considered in mammalian systems, are the effects of CM on membrane phosphorylation. In red blood cells, the active transport of Ca^{+2} from the cells is accomplished by a membrane bound (Ca^{+2}-Mg^{+2})-ATPase. An activator of the (Ca^{+2}-Mg^{+2})-ATPase was identified by Bond and Clough (1973) and later was shown to be a small, acidic, heat stable protein (Luthra, et al 1976). Other researchers (Gopinpath and Vincenzi, 1977; Jarret and Penniston 1977) had described the mimetic effect of CM on the activation of the (Ca^{+2}-Mg^{+2})-ATPase when compared to the "activator". Later, Jarret and Penniston (1978) demonstrated that the activator and CM were in fact identical. They also showed that CM increased the maximal Ca^{+2} activation and the affinity of the enzyme for Ca^{+2}. Niggli, et al (1979) have isolated the ATPase using a CM-sepharose column and the enzyme

was found to have molecular weight 125000 daltons. Its phosphorylation depended on the presence of Ca^{+2}. Hinds, et al (1978) also showed that CM stimulated Ca^{+2} transport implying that CM also affects the Ca^{+2} pump directly. The mechanism for the increased pump activity may be related to the increased phosphorylation of the pump ATPase noted by Niggli, et al (1979).

Another membrane preparation which has been shown to increase Ca^{+2} pump activity in the presence of CM is the cardiac microsomal fractions. Katz and Remtulla (1978) demonstrated that CM could stimulate ATP-dependent Ca^{+2} transport in microsomal preparations enriched with sarcoplasmic reticulum. However, the manner in which CM leads to an increased stimulation of Ca^{+2} transport is not known.

In another system, namely synaptosomes from brain, Ca^{+2} dependent activation of a membrane bound protein kinase is thought to lead to the phosphorylation of certain proteins (Schulman and Greengard, 1978a). The activiting protein was shown to be calmodulin (Shulman and Greengard, 1978b). It was noted that this phosphorylation seemed to have particular importance to neurotransmitter release since a "protein fraction" was discovered which regulated both the Ca^{+2} dependent neurotransmitter release and the Ca^{+2}-dependent protein phosphorylation (De Larenzo and Freedman, 1978). Later, De Larenzo, et al (1979), illustrated, using synaptic vesicles, that the previously termed "protein factor" re-

sponsible for both Ca^{+2}-dependent neurotransmitter release and protein phosphorylation was CM. Their experiments demonstrated that the removal of the endogenous heat stable protein from synaptic vesicles abolished Ca^{+2}'s effect on neurotransmitter release and protein phosphorylation. If the protein vesicle extract (or CM) was added back to the system, Ca^{+2}'s effects were restored. Conditions which stimulated the depolarization-dependent uptake of Ca^{+2} and neurotransmitter release of intact synaptosomes were also found to stimulate the Ca^{+2}-CM dependent phosphorylation of specific proteins in vesicles. Mahler and Sorensen (1980) have shown that in the presence of Ca^{+2}-CM, the protein kinase dependent phosphorylation of more than 12 synaptic membrane proteins can be observed. The apparent molecular weights of these proteins are p42, p48, p50, p59, p64, p100 (doublet), p115, p155, p190, p195, p200, p205 and p220. These proteins have been grouped into different families based on their responses to Ca^{+2} and/or CM. The general conclusion has been that Ca^{+2}-CM dependent phosphoylation of these vesicles proteins will occur in the intact nerve terminal during neurotransmitter release.

It should be noted that the CM dependent phosphorylation of specific endogenous membrane proteins cannot be limited to brain. Membrane preparations from spleen, lung, skeletal muscle, heart, adrenal, and vas deferens also exhibit the same dependence (Schulman and Greengard, 1978). Wang and

Waisman (1979) suggested that the varying arrays of phosphorylated membrane proteins in different tissues enable CM to modulate different functions. The tissue distribution of protein substrate of the CM dependent protein kinases determines CM's tissue specific regulatory activity.

2.5.1.9 Other enzymes

These also have been other enzymes whose activity have been found to be modulated by CM but will only be mentioned briefly in this review. Plant NAD kinase is one such enzyme. Anderson and Cormier (1978) noted the Ca^{+2} dependence of the activator of pea seedlings and also described its similarity to CM. This similarity was based on its acidity, heat stability, Stokes radii, interactions with Tn-I, and its ability to activate PDE and NAD kinase.

Another important enzyme which has been found to be activated by CM is phospholipase A2. This enzyme catalyzes the deacylation of phosphoglycerides at the 2 position to produce a lysophosphatide and a free fatty acid such as arachidonic acid which is a precursor to prostaglandin synthesis. In platelets, CM stimulates phospholipase A2 activity, and it Cheung (1980) suggested that such stimulation may occur n other tissures. This enzyme requires Ca^{+2} regardless of the tissue source suggesting that CM indeed, may be involved in phospholipase A2 regulation. To compound the situation, cAMP has been found to affect the activity of this

enzyme whose reaction products, such a prostaglandins, are important mediators of cellular activity. Cheung (1980) suggested that by controlling the activity of cyclic nucleotides and prostaglandins, CM may be a molecular link between these two groups of regulators.

Lastly, Nagao, et al (1979) and Suzuki, et al (in preparation) have illustrated that CM activates guanylate cyclase in the protozoan, Tetrahymena pyriformis. Although it is not known whether CM activates mammalian guanylate cyclase, it is known that Ca^{+2} is required for the mammalian enzymes activity (Goldberg and Maddox, 1977).

2.5.1.10 Structural Proteins

There also have been indicators that CM is involved in regulating the activities of two structural proteins other than myosin which are tubulin and actin. During earlier studies on microtubules, Weisenberg (1972) noticed that the in vitro microtubule (MT) assembly required the presence of GTP and EGTA (a Ca^{+2} chelator). Because EGTA was required for polymerization, it was suspected that Ca^{+2} would inhibit this process and this was later confirmed. However the exact concentration of Ca^{+2} required varied with the study (Synder and McIntosh, 1976). Nishida and Sakai (1977) first demonstrated the Ca^{+2} sensitizing effects of a factor from porine brain extracts. Later, Marcum, et al (1978) showed that in the presence of stoichiometric amounts of CM or

Tn-C, tubulin polymerization was completely inhibited by 10uM Ca^{+2}. This concentration of Ca^{+2} only partially inhibited tubulin polymerization in the absence of CM or Tn-C. However, if lower Ca^{+2} concentrations were used (<1uM), no inhibition by the Ca^{+2} binding proteins, CM or Tn-C, was observed. It can be noted that as the concentration of Ca^{+2} increases from 0.1uM to 10uM (which are physiological), MT disassembly is favoured. Although Tn-C was found to mimic the effects of CM in this study, it is believed that CM is the physiological regulator of MT assembly in vivo. The reasons for this idea are:

1. Tn-C has not been identifed in any non-muscle tissue.
2. CM has been identified in various non-muscle sources where the concentration of tubulin is also high (Synder and McIntosh, 1976; Smoake, et al 1974).

Apart from affecting the assembly/disassebly of tubulin, or perhaps because it does affect this polymerization process, CM has also been found to affect various functions associated with tubulin. One process in which both CM and tubulin have been implicated is that of mitosis. Dedman, et al (1978)and Welsh, et al (1978) have shown that CM associates with the mitotic spindles composed largely of tubulin in a pattern mimicking the various stages of mitosis. This was shown using CM antibodies and indirect immunofloures-cence. In their studies, it was demonstrated that, with the onset of prophase, CM could be detected in a random distri-

bution throughout the cell. Later in prometaphase, CM was found associated with the half spindles of the mitotic apparatus. Throughout metaphase and most of anaphase, it was found in a region between the spindle poles and the chromasomes. In support of the view that CM is interacting with the tubulin component of the mitotic spindles during cell division is the observation that CM could be detectd in two regions where MT disassembly was known to occur, namely:

1. the half spindles during anaphase
2. the midbodies on either side of the interzonal cleavage furrow during telophase.

The previously mentioned experiments, which showed that CM causes a depolymerization of tubulin _in vitro_, support these _in vivo_ observations. Conversly, during prophase when MT's are being assembled, it is possible that the concentrations of Ca^{+2} are too low for CM to prevent the polymerization of tubulin. However, it is not necessary that CM be inactive during these early stages of cell division, since MT disassembly may in fact be required for the congressional movement of chromosomes.

Dedman, et al (1978) and Welsh, et al (1978) maintained that since CM is not found in the telophase cleavage furrow, whereas actin is concentrated in this area, CM was more likely to be associated with tubulin than actin. It was also suggested that CM only decorated actin filaments when they exist as stress fibres in resting cells. However, at a

later date, Welsh, et al (1979) discovered some subtle discrepancies between the localization of CM and tubulin. During early prophase, CM and tubulin fluorescence could be detected near the centrioles. Throughtout prometaphase and metaphase, the patterns of localization of the two proteins were again very similar. However during anaphase, differences in the localization of tubulin and CM were noted. Tubulin could be found dispersed within the interzone and in the area between the poles and the chromosomes. CM was also found between the poles and the chromosomes. However, its distribution within the interzone was different. CM was found in two distinct areas called midbodies which are found on either side of the interzone cleavage furrow during telophase. Lastly, in the two new daughter cells, the distribution of CM and tubulin were distinctly different. The researchers had also noticed that the similarities in the distributions of actin and CM were considerable. Based on these observations, they concluded that CM-actin interactions may be occurring during mitosis.

Welsh, et al (1979), also treated the Chinese hamster ovary cells with agents which would block the MT or micrfilament (MF) structure. When MT organization was disrupted with colcemid, both tubulin and CM specific flourescence were equally altered. If the cells were treated with cytochalasin B, a MF disruptor, no significant effect was observed. However, cell division was prevented. The idea

that actin is involved in cell division is supported by the finding of a number of researchers who showed that actin is thought to be a component of the half-spindles (Songer 1975; Cande, et al 1977; Schloss, et al 1979; Herman and Pollard 1978). Herman and Pollard (1979) have recently demonstrated that the localization of actin in the mitotic apparatus is very similar to that of CM found by Means and Dedman (1980). In their recent review, Means and Dedman (1980) proposed that CM may be activating the myosin light chain kinase to phosphorylate the LC(20). Upon phosphorylation of these light chains, a conformational change may take place which allows actin to activate the myosin ATPase. It is thought that this is at least one mechanism of motile behaviour in resting cells, but remains to be proven for dividing cells where chromosome movements are considered.

It is possible that both actin and tubulin are functioning during cell division at different times (Kiehart and Inove, 1976). However, these two proteins could be acting simultaneously with different responses to CM during mitosis. Another possibility is that actin and tubulin may be interacting with each other (Forer 1976) since they are very close within the mitotic apparatus.

The possibility that interactions between tubulin and actin exist is further supported by the <u>in vitro</u> findings of Griffiths and Pollard (1978), who showed that the polymerization of tubulin and actin together, in the presence of the

tau proteins, was greater than the polymerization of either protein individually.

It should be noted that the early role of CM in mitosis need not be restricted to regulating the polymerization of tubulin or to its interaction with actin and/or myosin. CM has been shown to regulate the activity of a Ca^{+2}-ATPase associated with the smooth endoplasmic reticulum (SER) which lies close to the half-spindles of mitotic cells (Harris 1975). It was suggested by Welsh, et al (1979), that CM could be functioning by regulating the Ca^{+2} concentrations of the half-spindles throughout the SER Ca^{+2} pump system as was found in muscle and in non-muscle cells (Harrris 1975). It is important to note that CM may be playing multiple roles in regulating and coordinating mitosis as was found for other cellular activities such as glycogen metabolism.

Added to the complexity of the role of CM in cellular function was the finding that antibodies to CM and one of its binding proteins (CMBP-I) are found associated with tubulin. At the electron microscope level, CM and CMBP-I were found associated with neuronal elements at post-syntaptic densities within neuronal somata and dendrites. Furthermore, in the area of the dendrites, CM and CMBP-I were bound to the post-synaptic density and to the microtubules. Due to the common localization of CM, CMBP-I and MT's, it has been suggested that CM and CMBP-I may function in regulating tubulin (Cheung, et al 1980). These observations suggest

the possibility that CMBP-I may also be regulating mitosis since tubulin has been implicated in this activity. However, the low concentration of CMBP-I found in most cells except brain, may negate this possibility.

2.5.2 Calmodulin Binding Proteins

The CM binding proteins actually refer to a group of proteins which can specifically interact with CM, have the ability to inhibit CM-activated enzymes and proteins, and are not known to have any other function (Wang, et al 1980). Classification of these proteins has been based on the Roman numeric system, e.g. calmodulin binding protein I is CMBP-I.

2.5.2.1 Calmodulin Binding Protein - I

The first CMBP was discovered because Wang and Desais(1976) found that partially purified preparations of CM-dependent cyclic nuclectide phosphodiesterase from bovine heart required less CM for activation than similar preparations from bovine brain. When the partially purified enzyme was placed on a Sephadex G-200 column, an inhibitor protein could be separated from the enzyme. It was shown that the presence of this inhibitor protein accounted for the decreased activation of the PDE from bovine brain. Another group of investigators (Klee and Krinks, 1978) independently discovered a PDE inhibitor and it now appears that the two inhibitors are both CMBP-I.

CMBP-I was first purified from bovine brain by Klee and Krinks (1978) and later by Wallace, et al (1979), and Sharma, et al (1979). Homogeneous protein was obtained only after a 300 fold purification and this observation has led to the idea that CMBP-I constitutes only 0.3% of the total protein in the extracts. Althought this is only an estimation, it has been shown that this inhibitor is the most abundant of all bovine brain proteins capable of associating in a Ca^{+2}-dependent manner with CM (Klee and Krinks, 1978; Sharma, et al 1979).

The molecular weight of the purified protein was determined to be about 85000 daltons and is thought to consist of 2 polypeptides of 60000 and 14500 daltons (Klee and Krinks, 1978; Wallace, et al 1979; Sharma, et al 1979). These two subunits have been designated α (60000 daltons) and β (14500 daltons) (Sharma, et al 1979). There have been some discrepancies reported in the literature as to the correct mass ratio of the subunits. Sharma maintains that this ratic is two β to one α while Klee and Wallace maintain that it is one:one. Further study is required before the proper stoichiometry of the subunits can be determined and before the existence of two different forms of the protein can be established.

The mechanism of inhibition of the CM-dependent PDE has been determined to a large extent and characterized. Wang and Desai (1977) have presented a reaction mechanism based

largely on experiments performed on a Sephadex-G-200 column. It was shown that when CMBP-I and CM were applied to the gel filtration column in the presence of EGTA, the two proteins eluted at positions corresponding to the elution volumes of the individual proteins. However in the presence of Ca^{+2}, both proteins eluted at the same position indicating the Ca^{+2} dependence for association of the two proteins. Conversely, PDE did not associate with CMBP-I either in the presence of EGTA or Ca^{+2}. This suggested that the mechanism of CMBP-I inhibiting PDE was by a binding of CM rather than PDE. To substantiate this belief, it was necessary to prove that a ternary complex between CMBP-I-CM and PDE did not form and in fact, no evidence for a ternary complex in the presence of Ca^{+2} has been found. The Ca^{+2} dependent interaction between CM and CMBP-I has been confirmed by other investigators using affinity chromatography on CM-sepharose-4B conjugates (Klee and Krinks, 1978; Wallace, et al 1978), anion exchange and disc gel electrophoresis (Sharma, et al 1979), density gradient centrifugaion and chemical cross-linking (Richmond and Klee, 1978). Given these observations, the following reaction mechanism has been proposed:

$$Ca^{+2} + \underset{\text{(inactive)}}{CM} \longleftrightarrow Ca^{+2}\text{-}CM \longleftrightarrow \underset{\text{(active)}}{Ca^{+2}\text{-}CM^{*}} \qquad (1)$$

$$Ca^{+2}\text{-}CM^{*} + \underset{\text{(inactive)}}{PDE} \longleftrightarrow Ca^{+2}\text{-}CM^{*}\text{-}PDE \longleftrightarrow \underset{\text{(active)}}{Ca^{+2}\text{-}CM\text{-}PDE^{*}} \qquad (2)$$

$$Ca^{+2}\text{-}CM^{*} + CMBP\text{-}I \longleftrightarrow Ca^{+2}\text{-}CM^{*}\text{-}CMBP\text{-}I \qquad (3)$$

This reaction has been supported though various kinetic studies. First, it was shown that only the CM-dependent PDE was inhibited as increasing concentrations of CMBP-I were added to the activated enzyme. This proved that it was only the CM-activated activtiy which was inhibited by the enzyme and not the basal enzyme activity. Furthermore, Ca^{+2} chelation was shown not to be involved in the mechanism of inhibitor inactivation since the addition of excess Ca^{+2} did not restore the activated activity (Wang and Desai 1976; Wallace, et al 1978). Further support for the idea that CMBP-I acts by binding CM has been obtained by following the inhibition of PDE under varying amounts of inhibitor. A linear relationship was found between the amount of CMBP-I used in the assays and the amount of CM required for a 50% activation. When greater concentraions of CMBP-I were used, more CM was required to reverse the inhibition and conversely, when higher concentrations of CM were used to activate the PDE, more CMBP-I was required for enzyme inhibition (Wang and Desai, 1977). These results suggested that CM and CMBP-I interact stoichiometrically in the PDE reaction. Despite these findings, there has been some controversy over the stoichiometry of the binding of CM to CMBP-I. Richmond and Klee (1978) maintain that the stoichiometry is 0.7:1 while Huang, et al (1978) have evidence that the stichiometry is 1:2. Whatever the precise binding characteristics, there is a general concencus that CM binds to the α-subunit

of CMBP-I (Richmond and Klee, 1978; Sharma, et al 1979). The β-subunit is thought to be required for the stability of the α-subunit (Wallace, et al 1978) and it does not affect the inhibitory activity of the α-subunit (Sharma, et al 1979).

It is thought that the proposed mechanism of inhibition of PDE by CMBP-I can be applied to the mechanism of inhibition of other CM-activated enzymes. CMBP-I has been found to inhibit brain adenylate cyclase (Wallace, et al, 1978; Wescott, et al 1979), myosin light chain kinase (Waisman, et al 1978), erythrocyte (Ca^{+2}-Mg^{+2})-ATPase (Larsen, et al 1978) and CM stimulated Ca^{+2} transport in the red blood cell membrane (Larsen, et al 1978). In these systems, the inhibition is also on the CM stimulated activity and not on the basal activities in congruence as was found for PDE. Part of the ability of CMBP-I to inhibit these CM-stimulated enzymes may originate from the fact that CM isolated from various sources appears to be very highly conserved. CM from many different tissues has been shown to interact antagonistically to CMBP-I from bovine brain suggesting that CM lacks tissue or species specificity (Wang and Desai, 1976; Larsen, et al 1978; Waisman, et al 1978; Taylor, et al 1979).

Despite the ability of CMBP-I to inhibit CM-activated PDE, it should be noted that other enzyme proteins have similar abilites (Dabrowska, et al 1978; Tam, et al 1979). This observation raises the question of whether or not

CMBP-I has enzymatic activity. Although it has been suggested that CMBP-I may interact solely with CM-stimulated enzymes, Sharma has estimated that the molar ratio of CMBP-I:CM in bovine brain to be about 1:10. Due to the low concentraion of CMBP-I, it is difficult to conceive of it inhibiting CM regulated activities other than the PDE reaction. Several enzymatic reactions have been outlined as putative activites for CMBP-I. ATPase, GTPase, cAMP and cGMP PDE, 5'-nucleotidase, adenylate cyclse, guanylate cyclase, and protein kinase using histone, casein, and phosphorylase as substrates have been tested and shown not to interact with CMBP-I (Wang and Desai, 1977; Klee and Krinks, 1978).

2.5.2.2 Calmodulin Binding Protein II

Another inhibitor protein of the CM dependent cyclic nucleotide PDE was discovered (CMBP-II) during the purification of CMBP-I (Sharma, et al 1978a). Both CMBP-I and CMBP-II are specific for CM-dependent PDE. However, the one major difference is that CMBP-I is heat labile (i.e. it loses all of its inhibiting activity to PDE after incubating in a PDE boiling water bath for 1 minute) and CMBP-II is heat stable because it can be boiled for 15 minutes with out loosing any inhibitory activity (Sharma, et al 1978b).

CMBP-II has been shown to exist in very small amounts in bovin brain since it is purified over 200000 fold from the crude brain extract. Although it is less concentrated than

CMBP-I, CMBP-II is 20 times more potent in inhibiting PDE. It is not known if this protein inhibits the other CM-activated enzymes, but it is known that this inhibitor is not the same as the heat stable inhibitors of cAMP dependent protein kinase of a phosphoprotein phosphatase (Sharma, et al 1978a). Also unlike CMBP-I, CMBP-II is thought to be a monomeric protein of 70000 daltons. As demonstrated for CMBP-I, CMBP-II shows no phosphorylytic enzymatic activity towards ATP, GTP, cAMP, AMP, or histones.

It has been shown that many other proteins which have not been purified or characterized exist in tissues such as bovine brain. CM-Sepharose-4B affinity columns have been used to isolate CMBP-I, CMBP-II, and PDE. However, if bovine brain extract is appied to the CM affinity column in the presence of Ca^{+2}, a number of proteins specifically adsorb and can be eluted with buffers containing EGTA. Proteins of molecular weights p225, p150, p80, p41, and p38 have been identified from these eluted fractions (Klee and Krinks 1978; Watterson and Vanaman, 1976). A protein of molecular weight 20000 daltons has been isolated (Desai, Sharma, and Wang, unpublished) which exhibits inhibitory activity towards CM-activated PDE but which does not have any known enzyme activity.

2.5.3 Troponin

Other proteins which have been to shown to bind CM are the subunit of the troponin complex. Amphlett, et al (1976) and Dedman, et al (1977) showed that CM could substitute for Tn-C to form a complex with Tn-T and Tn-I, and that this complex conferred Ca^{+2} sensitivity towards the actomyosin ATPase. However, the Ca^{+2} sensitivity conferred with CM was not as great (Dedman, 1977). At about the same time that it was discovered that CM could substitute for Tn-C in the complex of Tn-I+Tn-T, it was also demonstrated by Amplhett, et al (1976) that CM could form a binary complex with Tn-I, and that this complex was more effective than Tn-C + Tn-I in regulating the Ca^{+2} sensitivity of the actomyosin ATPase.

The sucess of CM in substituting for the Tn-C in the Tn complex is largely due to the homology between the two proteins. It was originally noted that Tn-C and CM had very similar physiochemical properties but were different proteins by Wang, et al (1975) and Stevens, et al (1976). Kretsinger (in press) further characterized their similarities when he placed CM, Tn-C, as well as parvalbumin myosin light chains and mammalian intestinal Ca^{+2} binding protein within the same family of homologous proteins.

CM and Tn-C, as well as the other members of this family have been suggested to contain regions called "E-F hand structures" a term coined to represent an area in parvalbumin which is homologous to two other regions in the same

protein (A-B,and C-D). The three-dimensional structure of the E-F region is similar to a right hand with its forefinger and thumb extended and the second finger clenched. This is the origin of the term, E-F hand. The E-F hand contains a Ca^{+2} binding loop with 12 amino acids and two adjacent α-helices so that this region of CM contains a total of 35 amino acids. Ca^{+2} ions were found to ligate to oxygen atoms of amino acids found within the loop and the structure as a whole is stabilized by apolar residues in the α-helices. The importance of the E-F hand originates from the proposal that it is the basic unit from which the proteins of the family have evolved (Kretsinger 1976). The numbers of E-F hands in the Tn-C and CM correlate with the number of Ca^{+2} binding sites, but this is not true for all members of the family.

Apart from the evolutionary backround, similarity between Tn-C and CM has been suggested by analysis of their amino acid composition. It has been shown that if residue one of CM is aligned with residue 8 of Tn-C and a gap of 3 amino acids is introduced corresponding to 88-90 in Tn-C is introduced, 50% of the amino acids are identical and of the substituted, more than 50% are conservative replacements. If identical residues and conservative replacements are considered, there is 77% sequence homology between Tn-c and CM (Vanaman, et al 1977).

CM and Tn-C also have very similar sedimentation constants, molecular weights, isoelectric point and ultraviolet absorption spectra (Watterson, et al 1976; Stevens, et al 1976; Dedman, et al 1977; Wang, et al 1975). Furthermore, CM may be able to replace Tn-C as an activator of PDE but the concentration required is two to three orders of magnitude greater (Dedman, et al 1977; Potter, et al 1977). Others, however, have not been able to show activation of this CM dependent PDE by Tn-C (Stevens, et al 1976; Wang, et al 1975; Klee 1977).

Chapter III

MATERIALS AND METHODS

3.1 MATERIALS

All chemicals used were reagent grade from Fischer Scientific Company, CanLab, or Sigma Chemical Company. Adenosine 3':5'-cyclic-monophosphate (cAMP) was obtained from Sigma.

Frozen muscle was obtained from Pel-Freeze.

Fresh bovine brains were obtained from East-West meat packers in Winnipeg.

The proteins 5'-nucleotidase, found in Crotalus atrox venom, bovine serum albumin, ovalbumin, and cytochrome C were purchased from Sigma.

3.2 METHODS

3.2.1 Chromatography

A calmodulin-sepharose 4B affinity column was prepared as outlined by Sharma, et al (1980).

3.2.2 Protein Purifications

3.2.2.1 Calmodulin Dependent cyclic nucleotidase phosphodiesterase

Pure enzyme was prepared in our laboratory as outlined by Sharma, et al (1980).

3.2.2.2 Calmodulin

Bovine brain calmodulin was purified according to the method of Sharma and Wang (1979).

3.2.2.3 CMBP-I

This inhibitor was purified according to the procedure outlined by Sharma, et al (1979) and later modified in the laboratory (unpublished).

3.2.2.4 Total CMBPs from Bovine Brain

A batch of CMBPs were prepared according to the following procedure:

1. Frozen bovine brains were homogenized in 2.0 ml/g with 20mM Tris, 1mM Mg-acetate, 1mM imidazole at pH 7.0 (Buffer A).
2. The homogenate was centrifuged at 10000g for 20 minutes.
3. The supernatants were saved and the pellets were resuspended in an equal volume of Buffer A (see above) and recentrifuged.
4. All supernatants were recombined and adjusted to 10mM BME and 0.1mM EGTA.
5. The combined supernatant solutions were applied to a 400 ml DEAE cellulose column.
6. The column was eluted with Buffer A (see above), plus 0.05M NaCl, 10mM BME, and 0.1mM EGTA until the effluent was free of phosphate.

7. The column was washed with 0.2M NaCl, 10mM BME, 0.1mM EGTA containing Buffer A and the eluted fraction was collected in batch (about 2 bed volumes).
8. The batch was made up to 0.2M NaCl and 0.25mM $CaCl_2$ and left overnight stirring at 4^0C.
9. The solution was applied to a CM affinity column which had been equilibrated with Buffer A containing 0.01mM $CaCl_2$ and 10mM BME. This was eluted in batch with Buffer A plus 0.2M NaCl, 0.01mM $CaCl_2$, and 10mM BME. The washing with the above buffer was continued until no protein was detected by the dye binding method of Bradford (1976).
10. When no protein was detected in the column effluent, the column was eluted with Buffer A containing 0.2M NaCl, 0.1mM EGTA, and 10mM BME and 12 ml were collected per tube.
11. The fractions containing the protein peak were pooled.
12. Sucrose was added to 10% and this solution was concentrated under pressure using an Amicon-PM-10 filter. The final protein concentration was 0.87 mg/ml.
13. This sample was then divided into 2.0 ml aliquots and frozen at -20^0C.

These samples represent the total CMBPs used in the experiments. For assays of CMBPs, the following additonal steps were performed:

14. A fraction of the total CMBPs was applied to a 1.0 ml affinity blue gel column in order to remove PDE since low ionic strength PDE binds to the affinity blue gel.

15. The batch CMBPs were applied to a column which had been equilibrated with Buffer A containing 0.1mM EGTA and 10mM BME.

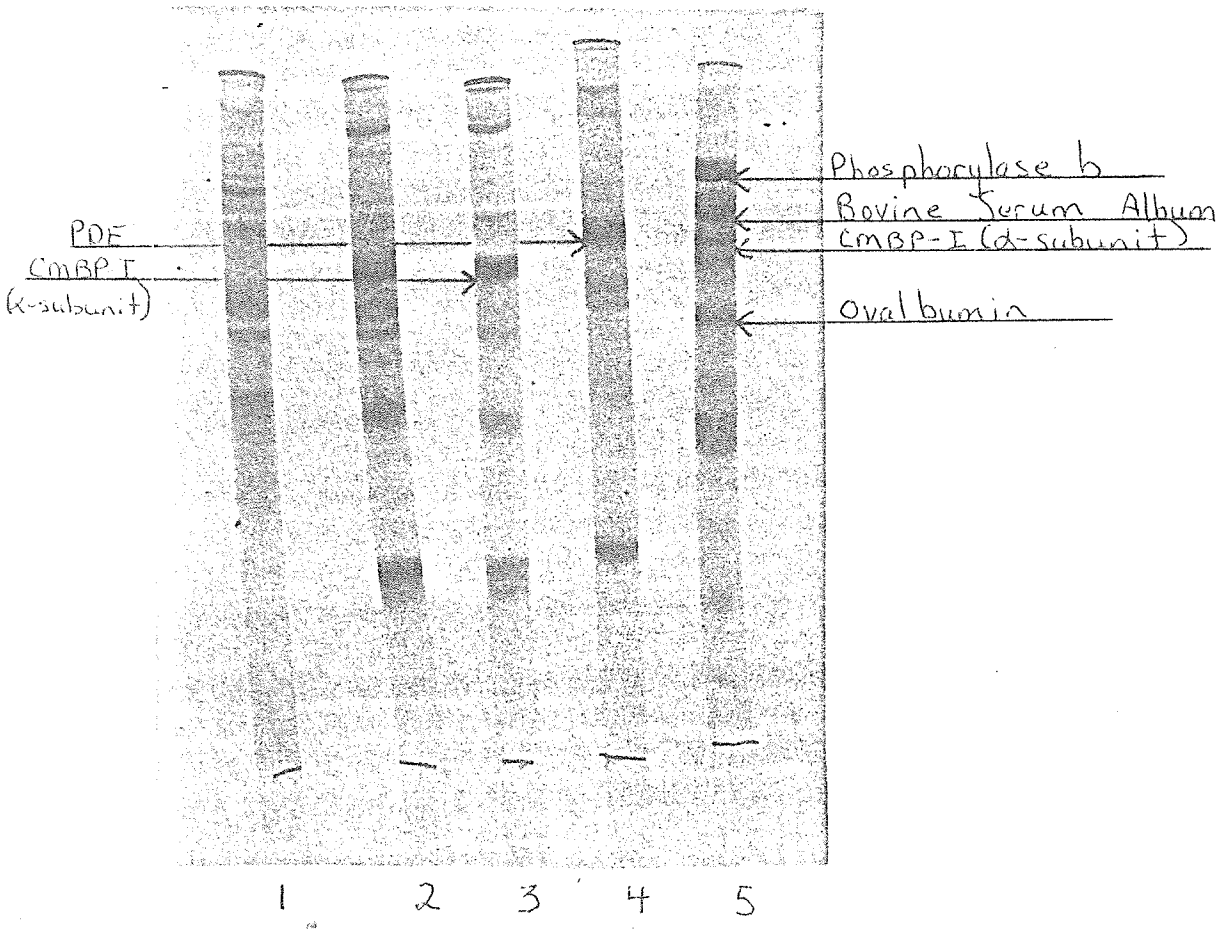
16. The column was washed with 3 bed volumes of Buffer A plus 0.2M NaCl, 0.1mM EGTA, and 10mM BME. A batch fraction was collected. This fraction contained little PDE as judged by SDS polyacrylamide gel electrophoresis and predominatly contained CMBP-I. For this fraction, one unit of PDE inhibitory activity (defined as that amount of CMPB which gives 50% inhibition of enzyme activated with 40 units of CM) was obtained with 0.39 ug.

17. The column was washed with Buffer A containing 1.5M NaCl, 0.1mM EGTA, and 10mM BME and a batch fraction collected. This fraction contained PDE as judged by SDS polyacrylamide gel electrophoresis and little CMBP-I. An assay of the CMBP fraction revealed that some PDE was contaminating this fraction since approximately 4 times more of this fraction was required when compared to pure CMBP-I (see the CMBP-I assay below). See Figure 1 for an illustration of the various fractions of CMBP-I assay.

From left to right:

1. Proteins from the 0.2M NaCl wash of DEAE cellulose
2. Total CMBPs from calmodulin affinity column
3. CMBPs from the 0.2M NaCl wash of an affinity blue gel column
4. Binding proteins from the 1.5M NaCl wash of the affinity blue gel column
5. Molecular weight marker

Figure 1: SDS-Urea polyacrylamide disc gel electrophoresis of CMBPs


Phosphorylase b
Bovine Serum Albumin
CMBP-I (α-subunit)
Ovalbumin
PDE
CMBP I
(κ-subunit)
1
2
3
4
5

3.2.2.5 Tropomyosin

This protein was prepared by the method of Greaser and Gergely (1971).

3.2.2.6 Phosphorylase b

Crystalline phosphorylase b was prepared from frozen rabbit muscles by the method of Fischer and Krebs (1958).

3.2.2.7 Actin

Actin was prepared from an acetone powder as outlined by Carsten and Mommaerts (1963) and was further processed by the method of Spudich and Watt (1971). The actin was extracted with 0.8M KCl as suggested by Gisenberg and Kielly (1974). Only one 0.8M KCl extraction was found to be necessary.

Table 1 illustrates the purification of actin from acetone powder. The extract of acetone powder refers to the supernatant obtained from the half hour extraction of actin from the acetone powder in G-actin buffer. The 0.8M KCl supernatant of the filtered acetone extract refers to the first supernatant obtained from the acetone extraction which has been filtered and treated with 50mM KCl and 2M $MgCl_2$ to polymerize the actin followed by the addition of KCl to 0.8M to remove the tropomyosin. This solution was then centrifuged and the resulting supernatant is the one noted by this heading. The pellet resulting from this centrifugation was

termed the 0.8M KCl pellet of the filtered acetone extract. This pellet is used to further purify the actin. After homogenization, the pellet was dialyzed for about three days to depolymerize the actin. The dialyzed solution was then ultracentrifuged at 110,000g for 1-2 hours to remove actin which would not depolymerize during the dialysis. The resulting supernatant was termed the "final supernatant" and was used in the studies that follow. In this particular purification, which was representative of all purifications performed, 5.0g of acetone powder yielded approximately 50.0mg of pure actin. The "final pellet" also refers to the pellet obtained during the last ultracentrifugation.

The purification of actin was followed by SDS-Urea polyacrylamide (7.5%) gel electrophoresis (Figure 2). The same titles were used in this figure as in Table 1 except some intermediate steps are included. Slot 1 in this figure refers to the extraction of actin from the acetone powder before centrifugation where as slots 2-5 refer to the supernatants and pellets obtained after centrifugation. Slots 6 and 7 refer to the supernatants of slots 2 and 3 after filtration. Slots 8-11 are as in table 1 and slots 12-15 are representing the final supernatants and pellets of table 1

As shown in Figure 2, the first acetone extract is quite pure and, by the time that extract has been treated with 0.8M KCl and centrifuged, the resulting pellet is very pure. The final supernatant, (slot 12 in Figure 2, gel 1 in Figure

TABLE 1

Purification of actin isolate from rabbit skeletal muscle

Stage of Purification	Protein Concentration (mg/ml)	Volume (ml)	Total Protein (mg)
Extract of acetone powder	4.400	34.00	149.600
.8mM KCl supernatant of filtered acetone extract	.985	38.00	37.430
.8mM KCl pellet of filtered acetone extract	2.310	23.00	53.130
Final supernatant	2.550	18.50	47.175
Final pellet	23.200	00.25	5.800

From left to right:

1) Extract of acetone powder (40ug).

2) Supernatant of acetone extract (20ug).

3) Supernatant of acetone extract (40ug).

4) Pellet of acetone extract (20ug).

5) Pellet of acetone extract (40ug).

6) Filtered supernatant of acetone extract (20ug).

7) Filtered supernatant of acetone extract (40ug).

8) 0.8M KCl supernatant of acetone extract (20ug).

9) 0.8M KCl supernatant of acetone extract (40ug).

10) 0.8M KCl pellet of filtered supernatant (20ug).

11) 0.8M KCl pellet of filtered supernatant (40ug).

12) Dialyzed supernatant of 0.8M KCl pellets (final superna (20ug).

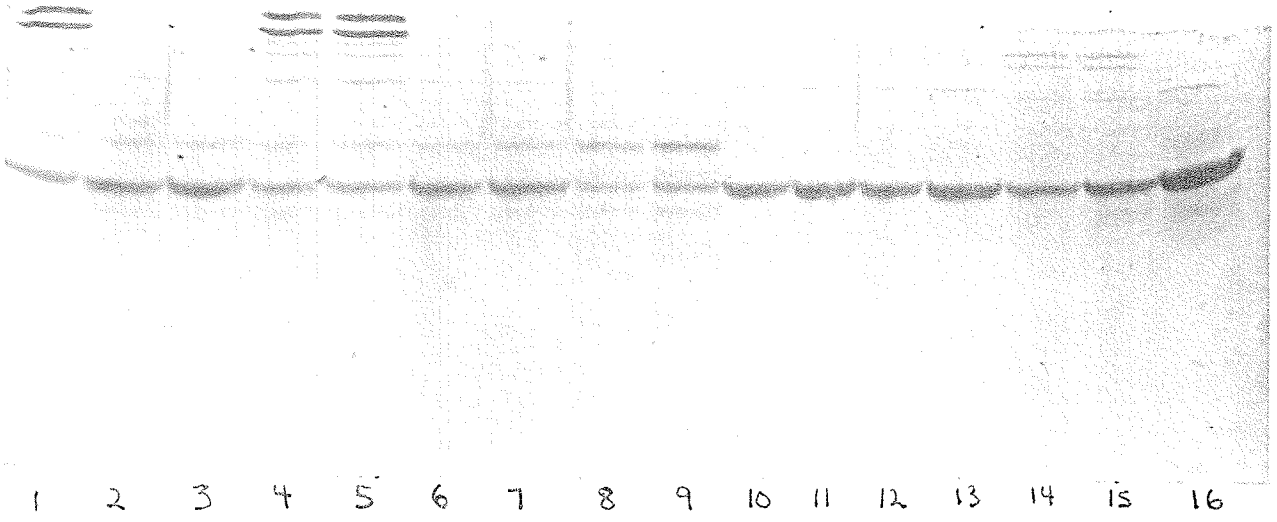
13) Dialyzed supernatant of 0.8M KCl pellets (final superna (40ug).

14) Dialyzed pellet of 0.8M KCl pellet (final pellet) (20u

15) Dialyzed pellet of 0.8M KCl pellet (final pellet) (40u

16) Actin marker (15ug).

Figure 2: SDS-Urea polyacrylamide slab gel electrophoresis of an actin preparation from rabbit skeletal muscle


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

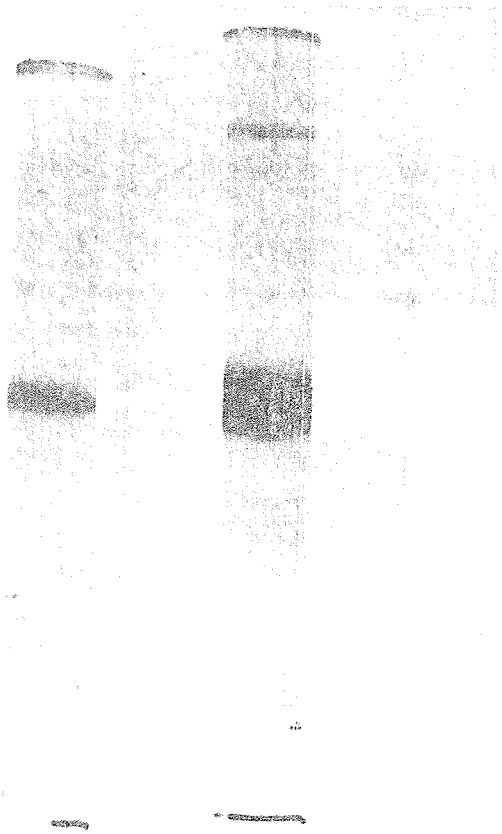
3) which is used for the experiments is very pure as judged by SDS-Urea polyacrylamide (7.5%) slab and disc gel electrophoresis.

Gel:

1) Actin supernatant (20ug) used in studies.

2) Actin pellet (40ug).

Figure 3: SDS-Urea polyacrylamide disc gel electrophoresis of an actin preparation



1 2

3.2.3 Assays

3.2.3.1 Phosphodiesterase assay

The activity of CM-dependent cyclic nucleotide PDE was determined by the method of Butcher and Sutherland (1962). This procedure involves the coupling of the PDE reaction with 5'-nucleotidase and the measurement of inorganic phosphate produced. The assay was carried out at 30°C, pH=7.5 in a reaction mixture containing 40mM Tris-HCl, 40mM Imidazole, 5mM Mg-acetate, 0.1mM $CaCl_2$, 1.2mM AMP, and the appropriate amount of CM (40 units).

3.2.3.2 Calmodulin assay

The ability of CM to activate CM-dependent cyclic nucleotide PDE was used in this assay. One unit of CM was defined as the amount of this protein required to produce 50% of maximum stimulation of PDE at 30°C as described previously (Teo, et al 1973).

3.2.3.3 CMBP-I assay

In this assay, two different preparations were used. One preparation was pure, however, the other was only partially purified as described above. The CMBP was tested for its inhibitory activity against CM-dependent PDE as outlined by Wang and Desai (1977). In this assay, the amount of CMBP leading to a 50% inactivation of maximally activated PDE was

defined as one unit of inhibitory activity. For pure CMPB-I, one unit=0.1 ug. For the total CMBP batch, one unit=0.39 ug.

3.2.4 Viscosity measurements

Viscosity was measured using a Cannon-Manning Semimicroviscometer size 75, after equilibrating samples for 15 minutes at 25^{0}C or 27^{0}C unless otherwise stated. This 15 minute equilibration period ensured that no air bubbles formed which would obstruct the capillary tubing of the viscometer. There were two basic types of viscosity experiments performed.

First, the ability of actin to polymerize was checked for each perparation of actin as follows:

1. Varying amounts of actin were placed in tubes at room temperature and the volumes were made up to a constant amount with G-actin buffer which contained 0.2mM $CaCl_2$, 0.2mM ATP, 10mM BME, and 2.0mM Tris at pH=8.0.
2. KCl and $MgCl_2$ were added to bring the final concentration to 50mM and 2mM respectively (conditions which induce G-actin to polymerize).
3. The tubes containing the varying amounts of actin were equilibrated at 4^{0}C overnight. It was necessary to equilibrate the polmerization process to ensure that a constant final steady state condition was

achieved. If the actin polymerization was in its inital reaction stages, the viscometer readings would be continually changing.

The specific viscosity was calculated as:

$$\eta_{sp} = \frac{\text{Time (sample)}}{\text{Time (G-actin)}} - 1$$

The second type of viscosity experiment performed, included those experiments where the steady state actin polymerization was measured in the presence of other proteins. For these experiments, the actin was either allowed to interact with the other proteins for about two minutes before adding the polymerizing agents (KCl and $MgCl_2$) or actin and other proteins were allowed to interact for two hours before adding KCl and $MgCl_2$. If the samples contained only CM and actin, the tubes were incubated at 25^0C. However, if CMBPs were present, it was necessary to incubate at 4^0C since they lose activity with time at room temperature. Those experiments where actin has interacted with other proteins for only a two minute incubation experiment are denoted as "Effects of Protein-x on Actin polymerization". Those experiments where actin was incubated with another protein for two hours, are denoted "Effect of protein-X on the G-actin to F-actin conversion". Samples were equilibrated after adding the polymerizing agents at 4^0C overnight. The specific viscosity was measured as before. Note that the presence of

the other proteins did not significantly change the viscosity of G-actin. However, in some cases, the reading obtained for G-actin in the presence of other proteins is used to calculate the specific viscosity.

3.2.5 Ultracentrifugation studies

In these experiments, actin was polymerized as described above, and various proteins were added. The following procedure was then performed:

1. The tubes were equilibrated overnight at $4^{0}C$.
2. The samples were ultracentrifuged at 110000g for 2-3 hours between $5^{0}C$ and $10^{0}C$.
3. The resulting pellets and supernatants were thoroughly separated.
4. The pellets were suspended in a constant volume of SDS-urea disruption buffer and the supernatants were dialized against water for about 8 hours.
5. The supernatants were frozen and lyophilized and prepared for SDS-Urea polyacrylamide slab gel electrophoresis.

3.2.6 SDS-Urea polyacrylamide gel electrophoresis

The Weber and Osborn (1969) method for preparing gels was used except urea was also included as suggested by Sender (1971). Instead of 5M urea, however, 6M urea was used. A 7.5% gel was made in the presence of 0.1% SDS. The slab gel

was stained and destained according to the method of Weber and Osborn. A constant current of 60 milliamperes was applied to the slab gel until the dye front had descended into the gel, at which point, the current was increased ot 200 milliamperes. The same methods were used for the SDS-urea polyacrylamide tube gels except 5 milliamperes was applied per gel until the dye front descended, followed by a constant 8 milliamperes per gel.

3.2.7 Protein concentration

The absorbancy at 280nm at concentrations of 1 mg/ml and light paths of 1 cm were assumed to be 1.11 for actin (Young, et al 1964).

The protein concentration for other proteins or mixtures of actin with other proteins was determined according to the Coomassie Blue dye binding method as developed by Bradford (1976).

Chapter IV

EXPERIMENTAL RESULTS

4.1 CHARACTERIZATION OF ACTIN

Two basic criteria were established to characterize actin. The first, purity, as described in Methods and Materials, was judged by SDS-Urea slab gel electrophoresis. The second criterion was based on the polymerization of actin. Each preparation of actin was checked for its ability to polymerize within certain limits. These limits are that a linear relationship should exist between specific viscosity and actin concentrations between 0.03 mg/ml and 0.9 mg/ml. Linearity is lost at concentrations higher than about 0.9 mg/ml (see Figure 4). To calculate the specific viscosity of F-actin, the following was used:

$$n_{(sp)} = \frac{\text{Time}_{(\text{F-actin})}}{\text{Time}_{(\text{G-actin})}} - 1$$

where time is in minutes and refers to the time required to flow through the viscometer. For each study, it was confirmed that G-actin (as actin exists in absence of 50mM KCl and 2mM $MgCl_2$) had a flow through time similar to that of buffer without any actin present (see Table 2 for experimental procedure).

Figure 4: Specific Viscosity vs. F-actin Concentration

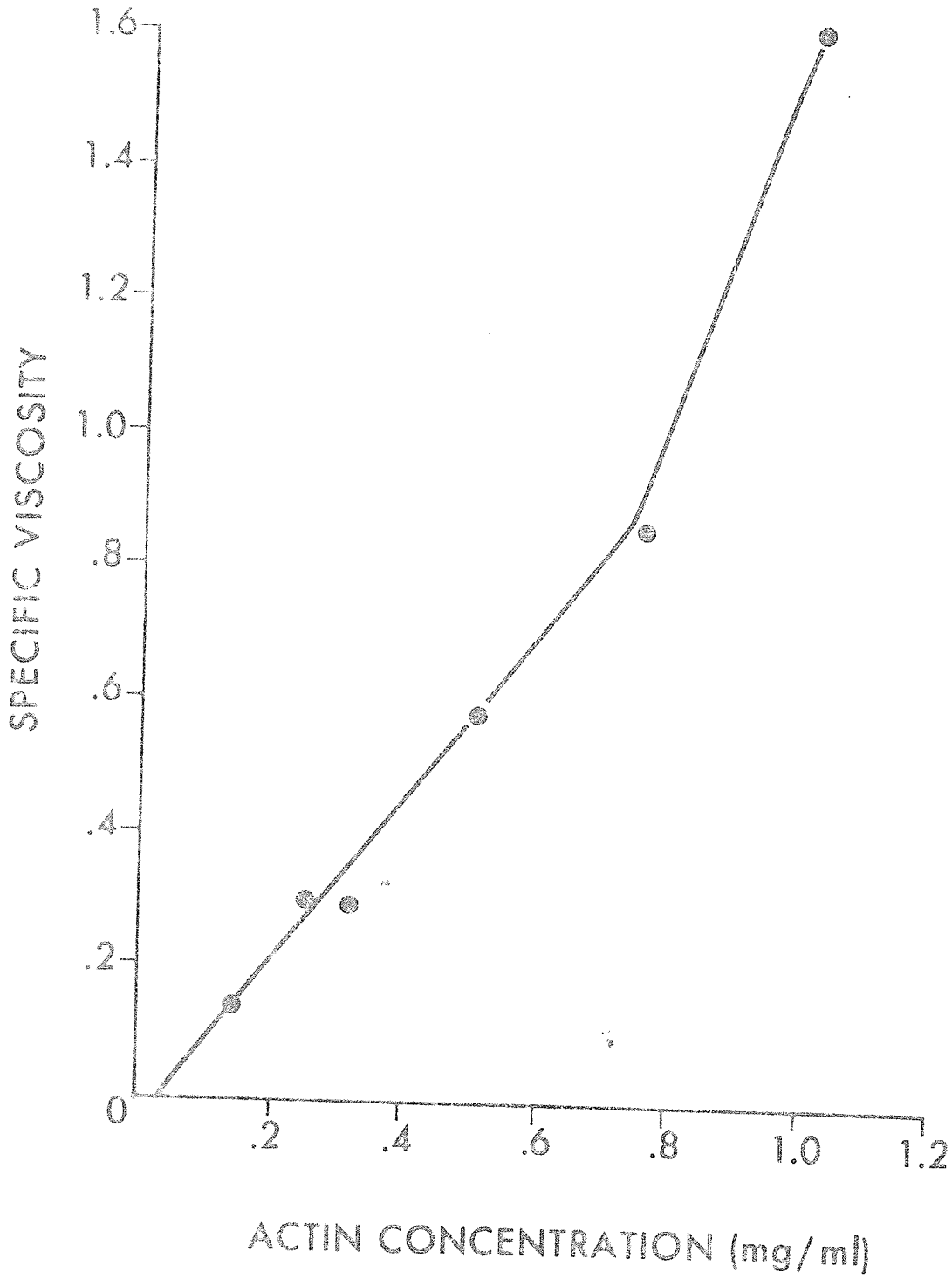

SPECIFIC VISCOSITY
1.6
1.4
1.2
1.0
.8
.6
.4
.2
0
.2
.4
.6
.8
1.0
1.2
ACTIN CONCENTRATION (mg/ml)

TABLE 2

Specific Viscosity of Different Concentrations of F-actin

Condition (reps)	Actin Concentration (mg/ml)	G-Actin Buffer (ul)	3.2mg/ml Actin (ul)	1500mM KCl + 60mM $MgCl_2$ (ul)	Ave. Time (min.)	Specific Viscosity n(sp)
Buffer (3)	-	1450	-	50	2.14	-
G-Actin (2)	1.07	1000	500	-	2.20	.03
F-Actin (3)	.15	1378	72	50	2.43	.14
F-Actin (3)	.25	1332	118	50	2.75	.29
F-Actin (3)	.50	1216	234	50	3.41	.59
F-Actin (3)	.75	1097	353	50	3.97	.86
F-Actin[1] (3)	1.07	950	500	50	5.57	1.60

[1] An Actin concentration > .90 mg/ml loses linearity.

The second criteria is based on the results obtained from a plot of specific viscosity versus actin concentration. If this plot produces a straight line, this line should transect the X-axis between 0.03 mg/ml and 0.07 mg/ml. The actin concentration at this point is the critical concentration (see Figure 4).

4.2 EFFECT OF CM ON ACTIN POLYMERIZATION

4.2.1 Viscosity experiments

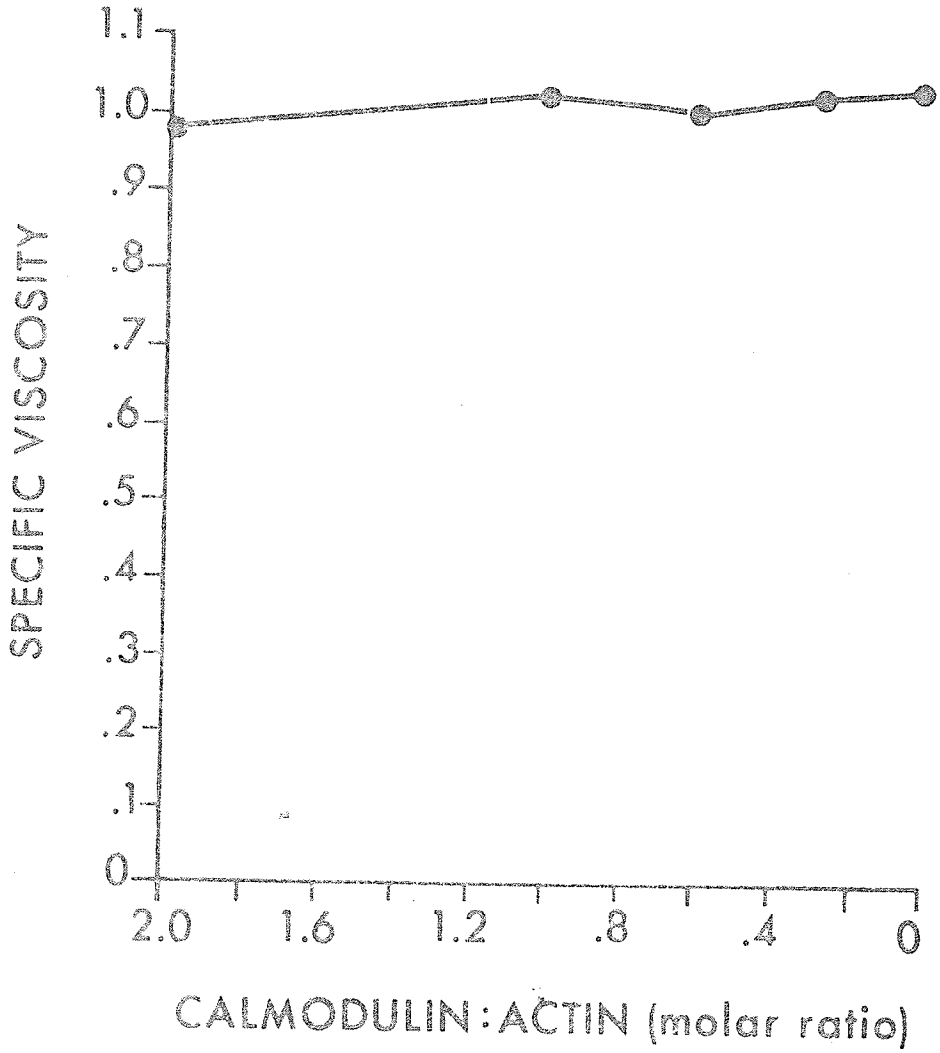
Various molar ratios of CM to actin were mixed with a fixed actin concentration of 0.84 mg/ml. This actin concentration was chosen because it was flexible in terms of detecting either an increase or decrease in polymerization using viscometry. After incubating the two proteins for a total of two minutes, 50mM KCl and 2mM $MgCl_2$ were added to the protein solution to induce polymerization.

As noted in Figure 5, molar ratios of CM to actin between 2:1 and 0:1 had no effect on actin polymerization at 27°C under the conditions described above. A 15 minute equilibration period at 27°C was used to remove all air bubbles as was also done for all succeeding experiments.

Although CM was shown to have no effect in the presence of Ca^{+2}, it was questioned whether CM would have an effect in the absence of Ca^{+2}. Therefore, the above experiment was repeated, except only 2 molar ratios of actin to CM were employed (5:3 and 5:5) to see if there was a trend in the ef-

The actin concentration was held constant at 0.84 mg/ml.

Figure 5: Effect of CM on actin polymerization in the presence of Ca^{+2}


SPECIFIC VISCOSITY
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
2.0
1.6
1.2
.8
.4
0
CALMODULIN : ACTIN (molar ratio)

fect of CM on actin polymerization in the presence and absence of Ca^{+2} (see Table 3). In this experiment, Ca^{+2} was chelated with EGTA, using approximately twice the concentration of EGTA as Ca^{+2}. The first five conditions in this experiment were controls which showed that buffer, G-actin, and CM alone had similar flow-through times. It was also shown that the presence or absence of Ca^{+2} did not affect the viscosity of F-actin. Furthermore, no effect of CM was observed on actin polymerization either in the presence or absence of Ca^{+2}. In this experiment, the specific viscosity was determined as:

$$n_{(sp)} = \frac{Time_{(sample)}}{(Time_{(G\text{-}actin)} + Time_{(CM)}) / 2} - 1$$

Note that the specific viscosity of G-actin and CM do not differ significantly. Therefore, for most of the following experiments the viscosities of samples are determined using G-actin alone.

The above experiment was repeated except actin and CM were allowed to interact for 2 hours before KCl and $MgCl_2$ were added to the samples (see Table 4). The first 8 conditions in this experiment are controls and it can be seen that there is no significant differences in flow-through times of buffer, G-actin with Ca^{+2}, CM with and without Ca^{+2}, or G-actin with CM in the presence and absence of Ca^{+2}. Also, the viscosity of F-actin was the same whether

TABLE 3

Effect of CM on Actin polymerization with and without Ca^{+2}

Conditions			Reps	Actin:CM (molar ratio)	G-Actin Buffer .2mM Ca^{+2} (ul)	G-Actin Buffer .5mM EGTA (ul)	1500mM KCl + 60mM $MgCl_2$ (ul)	Ave. Time (min)	Specific Viscosity n(sp)
Actin	CM	Ca^{+2}							
-	-	-	3	0:0	725	-	25	2.12	-
G	no	yes	3	5:0	375	-	-	2.10	.00
-	yes	yes	2	0:3	660	-	-	2.36	-
F	no	yes	2	5:0	350	-	25	3.95	.86
F	no	no	3	5:0	-	350	25	3.97	.87
F	yes	yes	2	5:3	260	-	25	4.19	.88
F	yes	no	2	5:3	-	260	25	4.14	.86
F	yes	yes	2	5:5	200	-	25	4.13	.85
F	yes	no	1	5:5	-	200	25	4.08	.83

or not Ca^{+2} was present. No trend in the effect of CM on actin polymerization was noted using three molar ratios of actin to CM (5:2, 5:5, 5:10) either in the presence or absence of CM. To calculate the specific viscosity of these samples, the following formula was used:

$$n_{(sp)} = \frac{Time_{(sample)}}{(Time_{(G\text{-}actin)} + Time_{(CM)}) / 2} - 1$$

TABLE 4

Effect of CM on the G-Actin to F-Actin conversion in the presence and absense of Ca^{+2}

Conditions				Actin:CM (molar ratio)	G-Actin Buffer withot Ca^{+2} without EGTA (ul)	10mM Ca^{+2} (ul)	10mM EGTA (ul)	H_2O (ul)	1500mM KCl + 60mM $MgCl_2$ (ul)	Ave. Time min.	Specific Viscosity n(sp)
Actin	CM	Ca^{+2}	EGTA								
-	-	-	-	0:0	1426	-	-	24	50	2.36	-
G	-	yes	-	5:0	1106	24	-	50	-	2.54	-
F	-	yes	-	5:0	1106	24	-	-	50	4.70	.85
F	-	-	yes	5:0	1106	-	13	11	50	4.69	.85
-	yes	yes	-	0:5	1176	24	-	-	50	2.37	-
-	yes	-	yes	0:5	1176	-	13	11	50	2.35	-
G	yes	yes	-	5:5	930	24	-	50	-	2.41	-
G	yes	-	yes	5:5	930	-	13	61	-	2.42	-
F	yes	yes	-	5:2	1006	24	-	-	50	4.73	.86
F	yes	-	yes	5:2	1006	-	13	11	50	4.67	.84
F	yes	yes	-	5:5	930	24	-	-	50	4.54	.79
F	yes	-	yes	5:5	930	-	13	11	50	4.48	.76
F	yes	yes	-	5:10	606	24	-	-	50	4.86	.90
F	yes	-	yes	5:10	606	-	13	11	50	4.57	.80

4.2.2 Centrifugation studies

Samples were prepared for these studies in the same manner as they were for viscosity studies, except that instead of placing the samples in the viscometer, they were ultracentrifuged. Figure 6 illustrates the results obtained after actin and CM have interacted for two minutes in the presence of either 0.2mM $CaCl_2$ or 0.01mM $CaCl_2$ (low Ca^{+2} in this figure). Slots (b-g) represent the pellets obtained from this centrifugation at 110000g for 3 hours at approximately 10^0C. Each pellet had been exposed to a certain amount of CM in the presence of normal (0.2mM) or low Ca^{+2}/ The pellets were suspended in a constant amount of SDS-Urea disruption buffer and constant amounts were applied to the gel. Note that there is no CM associated with the pellets at any concentration of $CaCl_2$ and that the relative intensity of staining of the pellets was similar in all slots suggesting that the CM did not depolymerize the F-actin.

The supernatants were dialyzed aginst deionized water overnight and then lyophilized. The protein was then suspended in disruption buffer so that the final concentration was about 1.0 mg/ml and a constant amount was applied to each gel. Note there is almost no actin in the supernatants again suggesting that CM did not depolymerize F-actin. All the CM was found in the supernatant suggesting that it had not bound to the F-actin in the pellets.

Samples were ultracentrifuged at 110,000g and the pellets and supernatants separated to note the location of calmodulin.

From right to left:

a) Actin marker (20ug)

b-g) F-actin pellets in 0.2mM Ca^{+2} or low Ca^{+2}

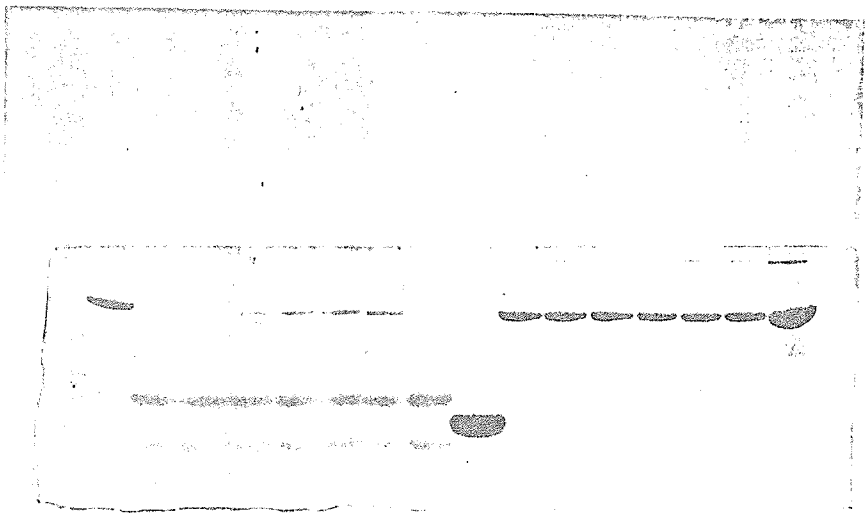
h) Cytochrome C marker (30ug)

i) Calmodulin marker (40ug)

j-o) F-actin supernatants corresponding to pellets b-g respectively in 0.2 mM Ca^{+2} or low Ca^{+2}

p) Tropomyosin marker

Figure 6: SDS-Urea polyacrylamide slab gel electrophoresis illustrating the effect of Calmodulin on G-actin polymerization



P	O	N	M	L	K	J	I	H	G	F	E	D	C	B	A	
-	5/10	5/10	5/5	5/5	5/3	5/3	-	-	5/10	5/10	5/5	5/5	5/3	5/3	-	Actin/Cm (molar ratio)
-	+	-	+	-	+	-	-	-	+	-	+	-	+	-	-	.2mM ($CaCl_2$)
-	+	-	+	-	+	-	-	-	+	-	+	-	+	-	-	.1mM ($CaCl_2$)

The above experiment was repeated (Figure 7) except the actin and CM were incubated for 2 hours before adding KCl and $MgCl_2$. Also in this case, some samples had EGTA to chelate the Ca^{+2}, and all the supernatants were made up with constant volumes so that any trend in the effect of increasing amounts of CM could be noticed. However, once again, no effect of CM on actin depolymerization was observed.

a) G-actin supernatant (about 25ug)

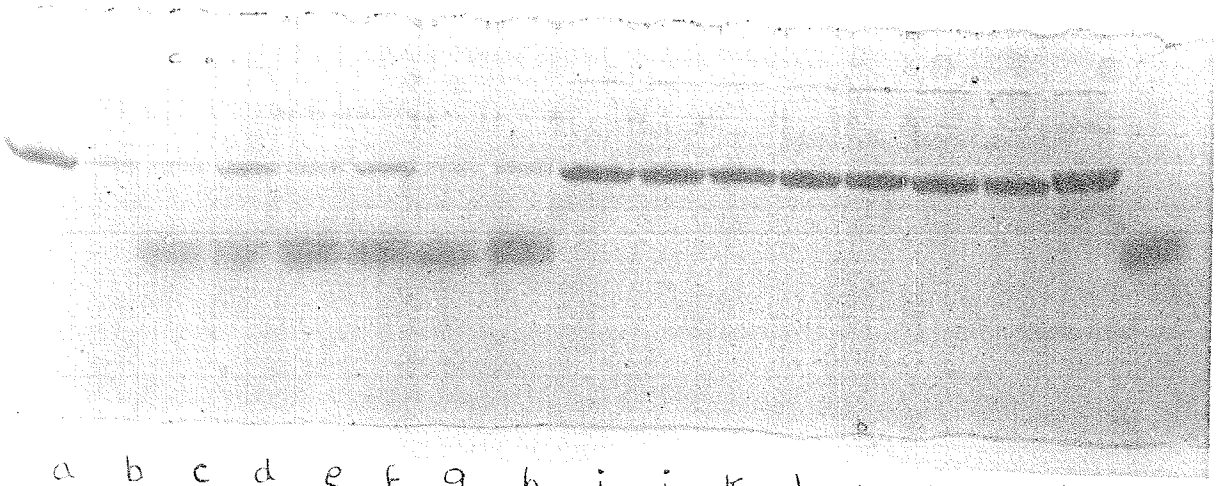
b-i) F-actin supernatants (about 25ug)

j-o) F-actin pellets (about 25ug)

p) Actin marker (20ug)

q) Calmodulin marker (25ug)

Figure 7: SDS-Urea polyacrylamide slab gel electrophoresis illustrating the effect of CM on the G-actin to F-actin conversion in the presence and absence of Ca^{+2}



a	b	c	d	e	f	g	h	i	j	K	l	m	n	o	p	q	
–	–	5	5	5	5	5	5	–	5	5	5	5	5	5	–	–	Actin (molar ratio)
–	–	4	4	6.7	6.7	13.3	13.3	–	4	4	6.7	6.7	13.3	13.3	–	–	cm
–	–	+	–	+	–	+	–	+	+	–	+	–	+	–	–	–	$CaCl_2$

4.3 EFFECT OF TOTAL CMBPS ON ACTIN POLYMERIZATION

4.3.1 Viscosity studies

A constant amount of actin was incubated for 2 hours with increasing amounts of the total CMBPs in the presence of Ca^{+2} under the conditions outlined in Tables 5 and 6 at 25^0 and 4^0C respectively. All procedures were identical to those previously outlined for viscosity studies with 2 hour incubation periods except that the incubation was only performed at 4^0C to preserve the integrity of the CMBPs. Also, for the study in Table 6, readings were taken at 4^0C. In Table 5, the first four conditions are controls showing that there is no significant difference in the specific viscosity of the buffer, G-actin, or the CMBPs alone, suggesting that there was no endogenous polymerization. The last three conditions in this table show that there was no trend in the effect of the increasing amounts of total CMBPs on actin polymerization at 25^0C, since there was no significant change in the specific viscosity values. The same controls and experimental conditions were used for the study shown in Table 6, and no consistent trend could be observed at 4^0C.

The experiment outlined in Table 5 was repeated except the presence of CM was included amoung some of the experimental conditions (see Table 7). According to the results of this study, the CMBPs did not have an effect on actin polymerization either in the presence or absence of CM with

TABLE 5

Effect of total CMBPs on the G-actin to F-actin conversion in the presence of Ca^{+2}

Condition	Reps	Actin Final conc. (mg/ml)	CMBP's Final conc. (mg/ml)	1500mM KCl + 60mM $MgCl_2$ (ul)	CMBP Buffer (ul)	Ave. Time (min)	Specific Viscosity n(sp)
Buffer -	2	-	-	50	1450	2.17	-
G-Actin -	2	.84	-	-	1015	2.18	-
- CMBP'S	3	-	.288	50	550	2.20	-
F-Actin -	3	.84	-	50	965	4.18	.92
F-Actin+CMBP's	2	.84	.064	50	765	4.09	.88
F-Actin+CMBP's	4	.84	.160	50	465	4.20	.93
F-Actin+CMBP's	2	.84	.288	50	65	4.17	.91

TABLE 6

Effect of CMBPs on the G-actin to F-actin conversion in the presence of Ca^{+2} at 4^0C

Condition	REPS	Actin Final conc. (mg/ml)	CMBP's Final conc. (mg/ml)	1500mM KCl + 60mM $MgCl_2$ (ul)	CMBP Buffer (ul)	Ave. Time (min.)	Specific Viscosity n(sp)
Buffer -	2	-	-	50	1450	3.735	-
G-Actin -	3	.84	-	-	1015	3.620	-
- CMBP's	2	-	.324	50	550	3.595	-
F-Actin -	4	.84	-	50	965	7.925	1.19
F-Actin+CMBP's	3	.84	.072	50	765	7.780	1.15
F-Actin+CMBP's	4	.84	.180	50	465	8.100	1.24
F-Actin+CMBP's	4	.84	.324	50	65	7.665	1.12

Ca^{+2}. This experiment was repeated in the presence of 0.09mM EGTA to chelate the 0.04mM Ca^{+2} (see Table 8) and again no consistent trend in the effects of the CMBPs on actin polymerization with or without CM was noted.

TABLE 7

Effect of total CMBP's on the G-Actin to F-Actin conversion with and without CM in the Presence of Ca^{+2}

Condition	REPS	Actin:CM G-Actin Buffer $+Ca^{+2}$ molar ratio	G-Actin Buffer + Ca^{+2} (ul)	CMBP's final conc. (ul)	CMBP's Buffer (ul)	1500mM KCl + 60mM $MgCl_2$ (ul)	10mM Ca^{+2} (ul)	H_2O (ul)	Ave. Time min.	Specific Viscosity n(sp)
Buffer - -	2	0:0	468	-	250	25	7	-	2.41	-
G-Actin - -	2	5:0	308	-	250	-	7	25	2.58	-
F-Actin - -	2	5:0	308	-	250	25	7	-	4.71	.83
F-Actin CM -	3	5:5	183	-	250	25	7	-	4.57	.77
F-Actin - CMBP's	3	5:0	308	.013	232	25	7	-	4.64	.80
F-Actin CM CMBP's	3	5:2	258	.013	232	25	7	-	4.70	.82
F-Actin - CMBP's	2	5:0	308	.033	205	25	7	-	4.61	.79
F-Actin CM CMBP's	2	5:5	183	.033	205	25	7	-	4.68	.81
F-Actin - CMBP's	3	5:0	308	.066	160	25	7	-	4.67	.81
F-Actin CM CMBP's	4	5:10	58	.066	160	25	7	-	4.61	.79

TABLE 8

Effect of total CMBP's on the G-Actin to F-Actin Converion with and without CM in the presence of EGTA

Condition	Reps	Actin:CM molar ratio	G-Actin Buffer without Ca^{+2} (ul)	.555 mg/ml CMBP's Final conc (mg/ml)	CMBP's Buffer (ul)	1500mM KCl + 60mM $MgCl_2$ (ul)	10mM EGTA (ul)	H_2O (ul)	Ave. Time min.	Specific Viscosity n(sp)
Buffer	2	0:0	468	-	250	25	7	-	2.37	-
G-Actin	2	5:0	308	-	250	-	7	25	2.58	-
F-Actin	3	5:0	308	-	250	25	7	-	4.64	.80
F-Actin CM	3	5:5	183	-	250	25	7	-	4.60	.78
F-Actin CMBP's	5	5:0	308	.013	232	25	7	-	4.50	.74
F-Actin CM CMBP's	2	5:2	258	.013	232	25	7	-	4.55	.76
F-Actin CMBP's	3	5:0	308	.033	205	25	7	-	4.62	.79
F-Actin CM CMBP's	4	5:5	183	.033	205	25	7	-	4.44	.72
F-Actin CMBP's	5	5:0	308	.066	160	25	7	-	4.39	.70
F-Actin CM CMBP's	4	4:10	58	.066	160	25	7	-	4.47	.73

4.3.2 Centrifugation studies

It was necessary to determine whether the total CMBPs were depolymerizing F-actin. This could have been noted by decreasing amounts of actin in pellets obtained by centrifuging as outlined in Materials and Methods, and as judged by SDS-Urea polyacrylamide gel electrophoresis. Also, if the total CMBPs were binding F-actin to cross-link it, they should have been detected in fractions associated with the pellets. To investigate these questions, G-actin and increasing amounts of the total CMBPs were incubated at $4^{0}C$ for 2 hours before adding the polymerizing agents. After allowing polymerization to equilibrate overnight, the samples were centrifuged and the pellets suspended in equal volumes of disruption buffer. Again no CMBPs were found associated with the pellets (see Figure 8) and there was no decrease in the amount of actin pelleted.

In a more complete experiment, the effect of total CMBPs on F-actin was checked with and without CM in the presence of Ca^{+2} (see Figure 9). In this study, the presence of the CMBPs and CM was verified in the supernatants but not in the pellets suggesting that these proteins are not binding to F-actin. There was no decrease in the amount of actin pelleted as increasing amounts of CMBPs with and without CM were added, suggesting that depolymerization was not occurring. To confirm this observation, there was no increase in the amount of actin found in the supernatants.

From left to right:

a) G-actin supernatant (about 25ug)

b) F-actin pellet

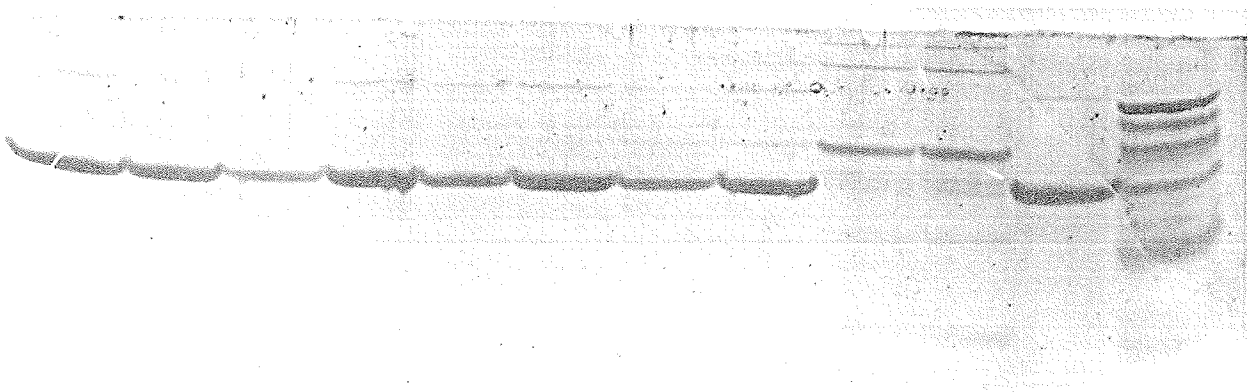
c-h) F-actin pellets with increasing amounts of CMBPs.
(c,e,g - about 15ug applied)
(d,f,h - about 30ug applied)

i-j) Total CMBPs (10ug and 20ug respectively)

k) Actin marker (15ug)

l) Molecular weight marker (15ug)

Figure 8: SDS-Urea polyacrylamide slab gel electrophoresis illustrating the effect of the total CMBPs on F-actin



a	b	c	d	e	f	g	h	i	j	k	l	
—	—	48	48	120	120	432	432	10	20	—	—	Total CMBPs (uc

From left to right:

a) F-actin pellet

b) G-actin pellet, CM, and total CMBPs

c-h) F-actin pellets with and without CM in the presence of CMBPs.

i) F-actin supernatant

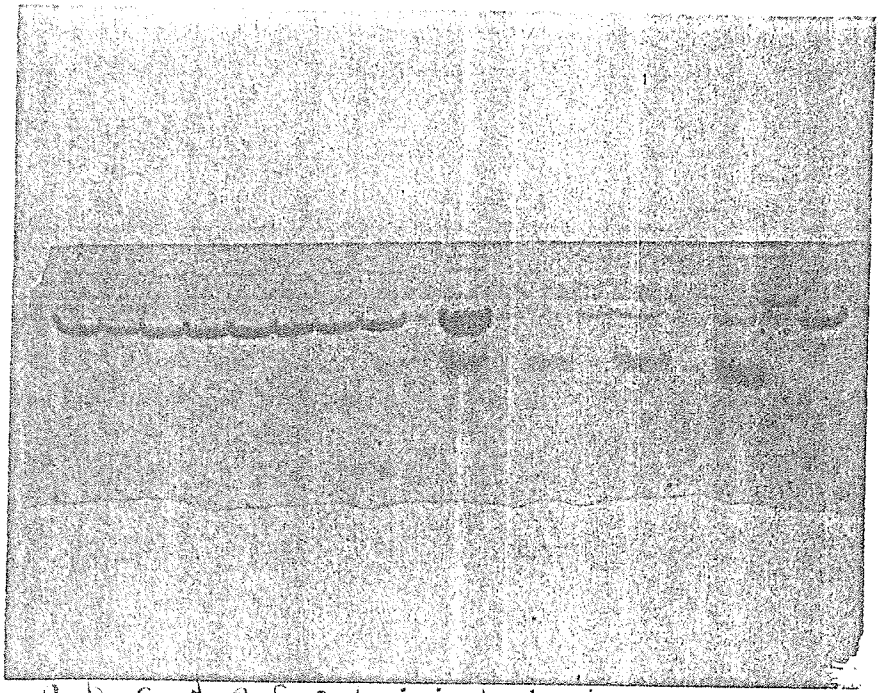
j) G-actin supernatant with CM and CMBPs

k-p) F-actin supernatants with and without CM in the presen of CMBPs corresponding to pellets (c-h) respectively

q) Total CMBPs
Note for (a-q) about 25ug was applied to each

r) Actin marker (15ug)

Figure 9: SDS-Urea polyacrylamide slab gel electrophoresis showing the effect of total CMBPs on F-actin with and without CM in the presence of Ca^{+2}



	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	
	5	5	5	5	5	5	5	5	-	5	5	5	5	5	5	5	-	-	Actin (molar ratio)
	0	5	0	2	0	5	0	10	-	5	0	2	0	5	0	10	-	-	CM
	-	50	20	20	50	50	90	90	-	50	20	20	50	50	90	90	-	-	Total CMBP's (ug.)

The above experiment was repeated except 0.27mM EGTA was included to clelate the 0.04mM $CaCl_2$, and, again no effect was seen on actin polymerization or on the binding of F-actin by the CMBPs or CM (see Figure 10).

From left to right:

a) F-actin pellet

b-g) F-actin pellets with and without CM in the presence of total CMBPs (about 25ug applied)

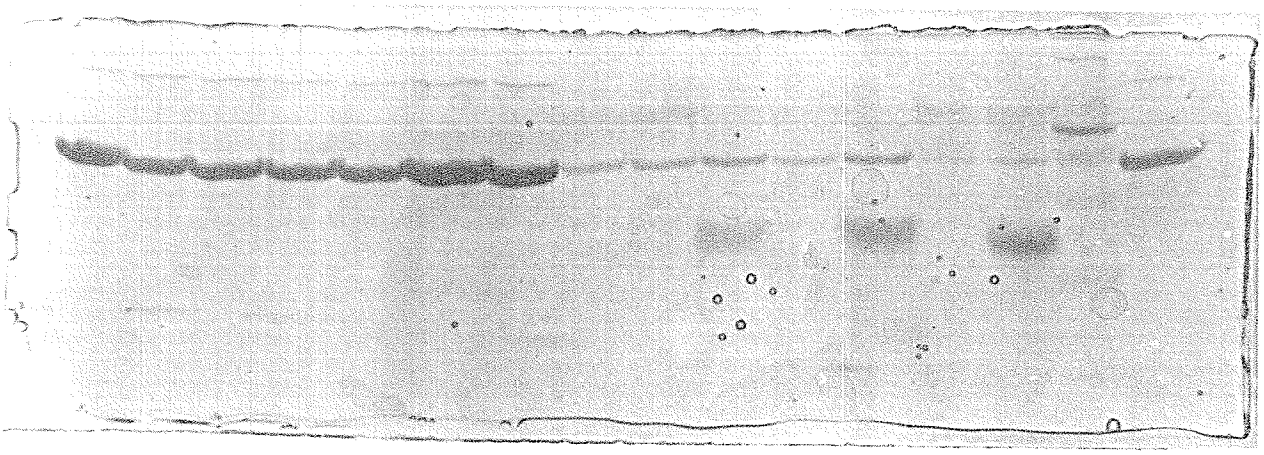
h) F-actin supernatant

i-n) F-actin supernatants with and without CM in the presence of the total CMBPs (about 10ug - 40ug applied)

o) Total CMBPs (20ug)

p) Actin marker (15ug)

Figure 10: SDS-Urea polyacrylamide slab gel electrophoresis illustrating the effect of total CMBPs on F-actin with and without CM in the presence of EGTA



a	b	c	d	e	f	g	h	i	j	K	l	m	n	o	p	
5	5	5	5	5	5	5	5	5	5	5	5	5	5	0	-	Actin (molar ratio)
0	0	2	0	5	0	10	0	0	2	0	5	0	10	0	-	CM
0	20	20	50	50	90	90	0	20	20	50	50	90	90	-	-	Total CMBPs (ug)

4.4 EFFECT OF CMBP-I ON ACTIN POLYMERIZATION

4.4.1 Viscosity studies

The effect of purified CMBP-I on actin polymerization was tested with a 2 minute incubation period using 0.168 mg/ml actin as shown in Table 9 . It should be noted that a 2 minute incubation was used and the experiment was done at 27°C. All other conditions were as previously outlined. The first 4 conditions in this study are controls which ensured that there was no endogenous actin or CMBP-I polymerization. The experimental conditions revealed that there was no effect of CMBP-I on actin polymerization using molar ratios of CMBP-I to actin of 1:10 through 2:10. One control was done in the presence of 0.40mM EGTA to chelate a maximum of 0.17mM $CaCl_2$, and the absence of Ca^{+2} did not affect the polymerization reading (see final condition).

Using higher actin concentrations (i.e. 0.84 mg/ml), and a 2 hour incubation period, no effect of CMBP-I on actin polymerization was noted between molar ratios of actin:CMBP-I between 5:0.04 and 5:0.2 (see Table 10). The first 8 conditions in this experiment are controls which revealed that the presence of CMBP-I does not affect the viscosity of G-actin with or without Ca^{+2}. Also, G-actin was shown to have no endogenous polymerization as evidenced by its similar viscosity to that found for the buffer. The last 6 conditions showed that increasing amounts of CMBP-I in the pres-

TABLE 9

Effect of CMBP-I on G-Actin Polymerization in the Presence of Ca^{+2}

Conditions CMBP-I:Actin molar ratio	Ca^{+2}	G-Actin Buffer (ul)	1500 mM KCl + 60mM $MgCl_2$ (ul)	Ave. Time (min)	Specific Viscosity n(sp)
0.00:00	yes	1450	50	2.23	.00
0.00:10	yes	1300	50	2.28	.02
1.00:00	yes	1300	50	2.19	.00
2.00:00	yes	1150	50	2.22	.00
1.00:10	yes	1150	50	2.28	.02
1.11:10	yes	1133	50	2.36	.06
1.25:10	yes	1112	50	2.30	.03
1.43:10	yes	1085	50	2.28	.02
1.67:10	yes	1050	50	2.25	.01
2.20:10	yes	1000	50	2.29	.03
1.43:10	no	*1085	50	2.41	.08

Notes:
Final concentration of Actin = .168 mg/ml.

* .4mM EGTA was in the buffer instead of .2mM $CaCl_2$

ence or absence of Ca^{+2} did not result in a consistent change in viscosity.

The above experiment was repeated in the presence and absence of CM and only in the presence of Ca^{+2} (see Table 11). Again, no trend in the specific viscosity of CMBP-I in the presence or absence of CM could be seen. This experiment was repeated in the presence of 0.05mM EGTA to chelate the 0.005mM Ca^{+2} (see Table 12). There was no observed effect of CMBP-I with or without CM in the absence of Ca^{+2} on actin polymerization using these conditions.

TABLE 10

Effect of CMBP-I on the G-Actin to F-Actin Conversion in the presence and absense of Ca^{+2}

Conditions	Reps	Actin: CMBP-I molar ratio	50mM EGTA (ul)	10ml Ca^{+2} (ul)	G-Actin Buffer -no Ca^{+2} -no EGTA (ul)	CMBP Buffer (ul)	H_2O (ul)	1500mM KCl + 60mM $MgCl_2$ (ul)	Ave. Time min.	Specific Viscosity n(sp)
Buffer	2	0:0	-	-	457	250	43	-	2.19	-
G-Actin Ca^{+2}	2	5:0	-	12	300	250	31	-	2.21	-
G-Actin EGTA	2	5:0	4	-	300	250	39	-	2.24	-
G-Actin Ca^{+2} CMBP-I	2	5:.10	-	12	300	125	31	-	2.22	-
G-Actin EGTA CMBP-I	2	5:.10	4	-	300	125	39	-	2.18	-
Ca^{+2} CMBP-I	2	0:.10	-	12	457	125	6	25	2.28	-
F-Actin Ca^{+2}	3	5:0	-	12	300	250	6	25	4.84	1.19
F-Actin EGTA	3	5:0	4	-	300	250	14	25	4.78	-
F-Actin Ca^{+2} CMBP-I	2	5:.04	-	12	300	200	6	25	4.62	1.08
F-Actin EGTA CMBP-I	2	5:.04	4	-	300	200	14	25	4.81	1.21
F-Actin Ca^{+2} CMBP-I	2	5:.10	-	12	300	125	6	25	4.80	1.16
F-Actin EGTA CMBP-I	2	5:.10	4	-	300	125	14	25	4.79	1.20
F-Actin Ca^{+2} CMBP-I	2	5:.20	-	12	300	-	6	25	4.54	1.04
F-Actin EGTA CMBP-I	1	5:.20	4	-	300	-	14	25	4.75	1.19

TABLE 11

Effect of CMBP-I on the G-Actin to F-Actin Conversion with and without CM in the Presence of Ca^{+2}

Condition	Reps	Actin: CMBP-I molar ratio	Actin:CM molar ratio	10mM Ca^{+2} (ul)	G-Actin Buffer (ul)	1500mM KCl + 60mM $MgCl_2$ (ul)	Ave. Time min.	Specific Viscosity n(sp)
Buffer	2	0:0	0:0	12	738	-	2.24	-
G-Actin	4	5:0	5:0	12	538	-	2.30	-
G-Actin CMBP-I	2	5:.1	5:0	12	413	-	2.30	-
G-Actin CMBP-I CM	3	5:.1	5:5	12	288	-	2.33	-
F-Actin	3	5:0	5:0	12	513	25	4.29	.87
F-Actin CMBP-I	3	5:.04	5:0	12	463	25	4.21	.83
F-Actin CMBP-I CM	9	5:.04	5:2	12	413	25	4.29	.84
F-Actin CMBP-I	5	5:.10	5:0	12	388	25	4.43	.93
F-Actin CMBP-I CM	11	5:.10	5:5	12	263	25	4.50	.93
F-Actin CMBP-I	3	5:.2	5:0	12	263	25	4.13	.80
F-Actin CMBP-I CM	7	5:.2	5:10	12	13	25	4.35	.87

TABLE 12

Effect of CMBP-I on the G-Actin to F-Actin Conversion with and without CM in the Presence of EGTA

Condition	Reps	Actin: CMBP-I molar ratio	Actin:CM molar ratio	10mM EGTA (ul)	G-Actin Buffer no Ca^{+2} (ul)	1500mM KCl + 60mM $MgCl_2$ (ul)	Ave. Time min.	Specific Viscosity n(sp)
Buffer	2	0:0	0:0	4	721	25	2.27	-
G-Actin	4	5:0	5:0	4	546	-	2.29	-
F-Actin	3	5:0	5:0	4	521	25	4.43	.93
F-Actin CMBP-I	2	5:.04	5:0	4	471	25	4.36	.90
F-Actin CMBP-I CM	4	5:.04	5:2	4	421	25	4.42	.93
F-Actin CMBP-I	4	5:.10	5:0	4	396	25	4.36	.90
F-Actin CMBP-I CM	2	5:.10	5:5	4	271	25	4.39	.92
F-Actin CMBP-I	2	5:.20	5:0	4	271	25	4.35	.90
F-Actin CMBP-I CM	3	5:.20	5:10	4	21	25	4.33	.89

4.4.2 Ultracentrifugation studies

The effect of CMBP-I on F-actin with and without CM was tested in the presence of Ca^{+2} (see Figure 11). In this study, increasing amounts of CMBP-I were incubated for 2 hours with F-actin with and without CM before polymerizing. The mixtures were equilibrated overnight at $4^{0}C$ and then centrifuged at 110000g for approximately 2 hours. Again CMBPs with or without CM were only found in the supernatants, suggesting that these proteins did not bind to the F-actin found in the pellets. Also, since the amount of F-actin in the pellets remained constant upon exposure to increasing amounts of CMBP-I and CM, it can be suggested that depolymerization was not occurring.

The above experiment was repeated in the presence of 0.5mM EGTA to chelate the 0.04mM $CaCl_2$ present, and again no effect on the F-actin was observed (see Figure 12).

From left to right:

a) F-actin supernatant

b) F-actinand CM supernatant

c-h) F-actin CMBP-I with and without CM supernatants (about 10ug - 75ug applied)

i) F-actin pellet

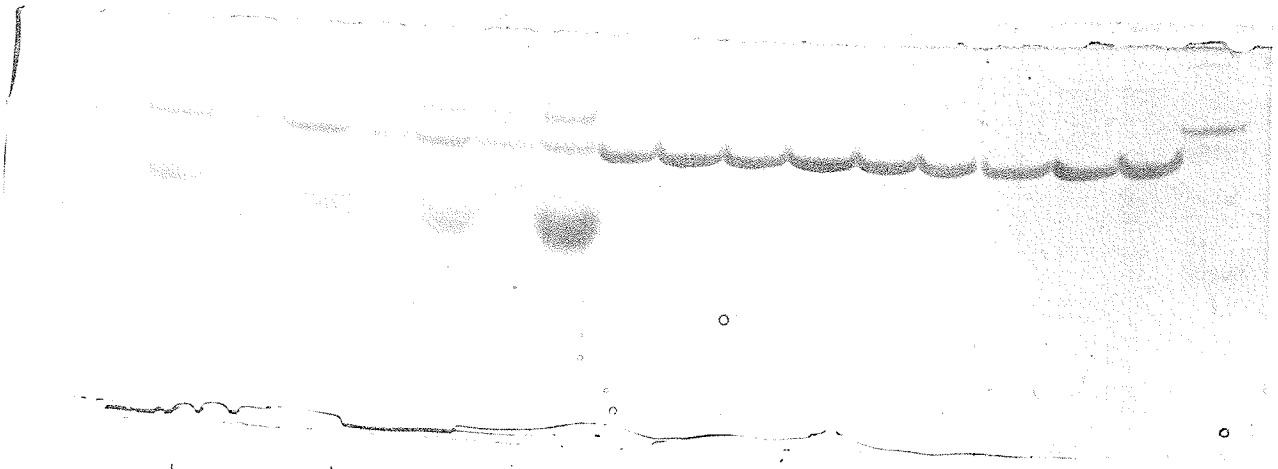
j) F-actin and CM pellet

k-p) F-actin and CMBP-I with and without CM pellets (about 30ug applied)

q) Actin marker (15ug)

r) CMBP marker (α-subunit)

Figure 11: SDS-Urea polyacrylamide gel electrophoresis showing the effect of CMBP-I on F-actin with and without CM in the presence of Ca^{+2}



a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	
5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	–	–	Actin / CM (mola ratio
0	5	0	2	0	4	0	10	0	5	0	2	0	4	0	10	–	–	
0	0	20	20	50	50	90	90	0	0	20	20	50	50	90	90	–	–	CMBP-I (uq

From left to right:

a) F-actin supernatant

b) F-actin and CM supernatant

c-h) F-actin and CMBP-I with and without CM supernatants (about 10ug - 75ug applied)

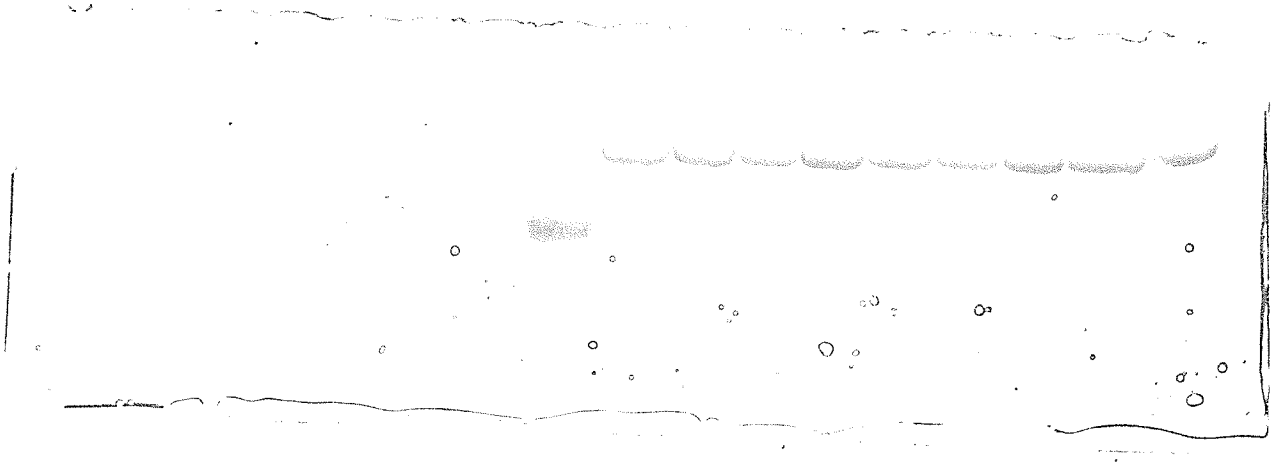
i) F-actin pellet

j) F-actin and CM pellet

k-p) F-actin and CMBP-I with and without CM pellets (about 30ug applied)

q) Actin marker (15ug)

Figure 12: SDS-Urea polyacrylamide slab gel electrophoresis showing the effect of CMBP-I on F-actin with and without CM in the presence of EGTA



a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	
5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	-	Actin (molar ratio)
0	5	0	2	0	4	0	10	0	5	0	2	0	2	0	10	-	CM
0	0	20	20	50	50	90	90	0	0	20	20	50	50	90	90	-	CMBP-I (ug.

4.5 CM-DEPENDENT CYCLIC NUCLEOTIDE PHOSPHODIESTERASE

In a preliminary experiment, actin was tested for its ability to activate phosphodiesterase (see Figure 13). Although there is a minor increase in the PDE activity with increasing actin concentrations, as evidenced by the increase in the OD(660), this could be attributed to some endogenous hydrolysis of ATP in the G-actin buffer. Actin was tested for the presence of CM in this lab, but none was found (unpublished observation).

It was then investigated as to whether actin may be binding the CMBPs as evidenced by a reversal of the CMBPs ability to inhibit PDE in the presence of Ca^{+2}. To explore this possibility, actin was added to the inhibited PDE 10 minutes after the reaction was initiated. Actin was unable to reverse the effects of the CMBPs at 1:1, 1:20 and 1:200 weight ratios of CMBPs to actin (see Figures 14, 15, and 16 respectively).

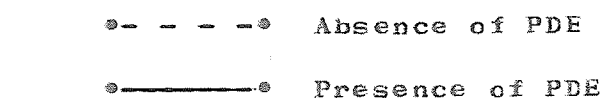
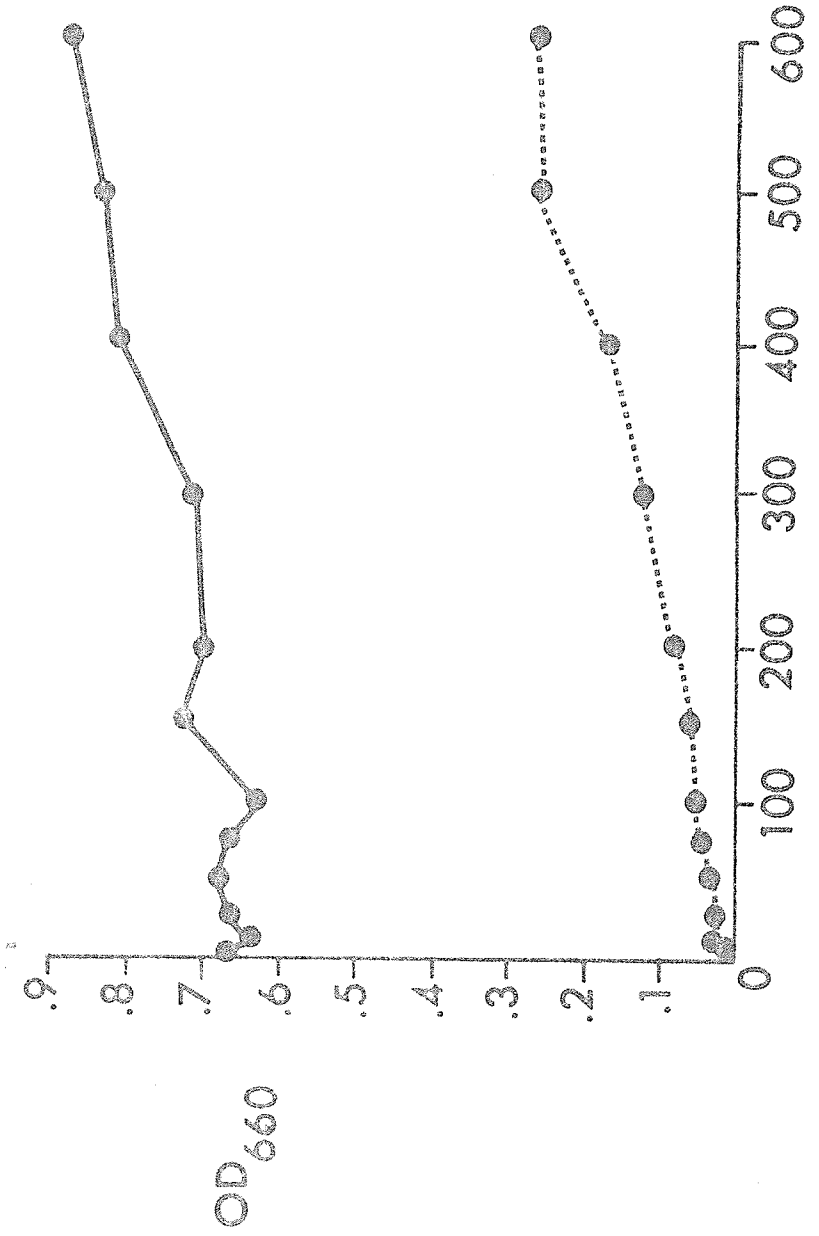


Figure 13: Test for the effect of actin on the PDE assay in the presence and absence of PDE


OD660
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
100
200
300
400
500
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VOLUME ACTIN (µl)
(3.68 mg./ml)

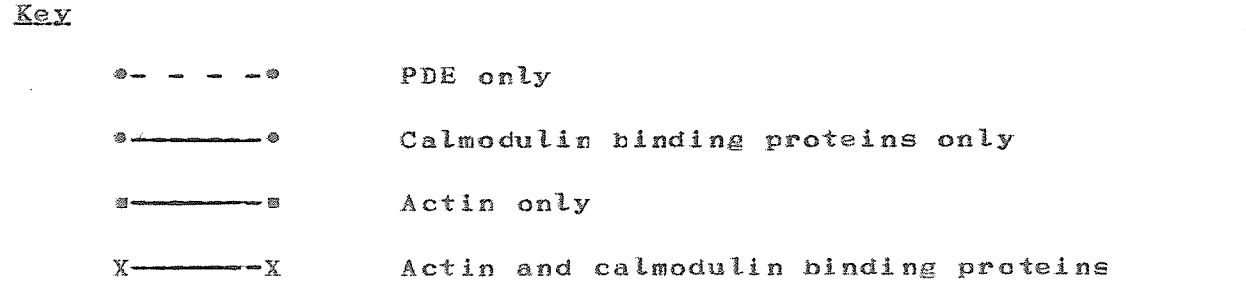
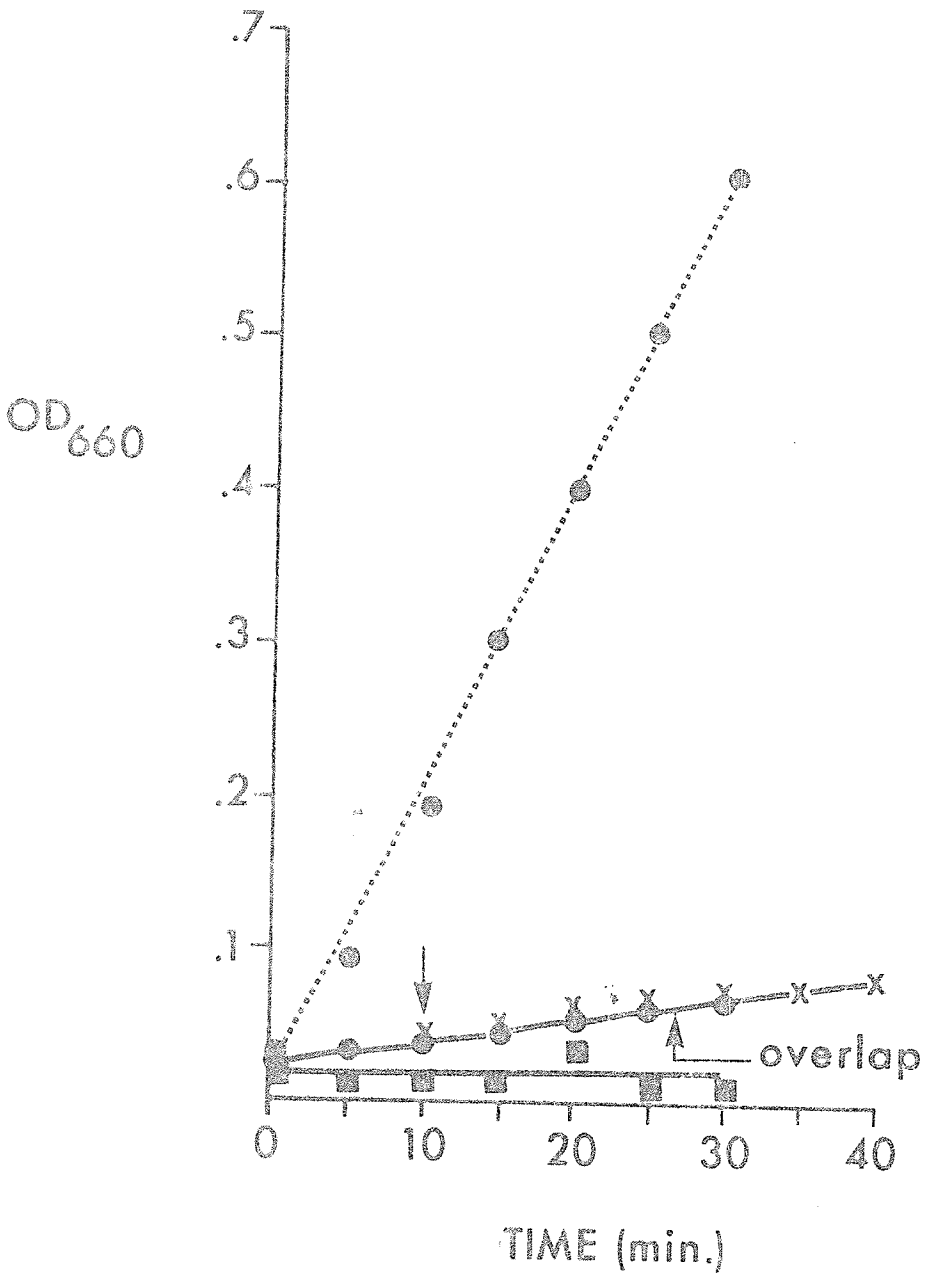


Figure 14: Test for the ability of actin to reverse the effects of the CMBPs on PDE using the PDE assay


.7
.6
.5
.4
.3
.2
.1
OD_{660}
overlap
0
10
20
30
40
TIME (min.)

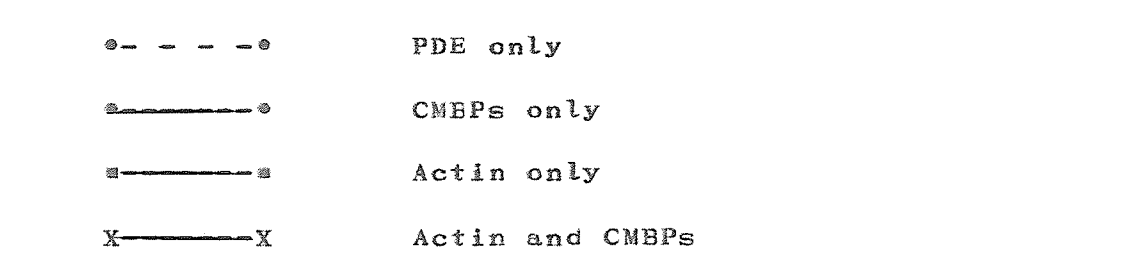
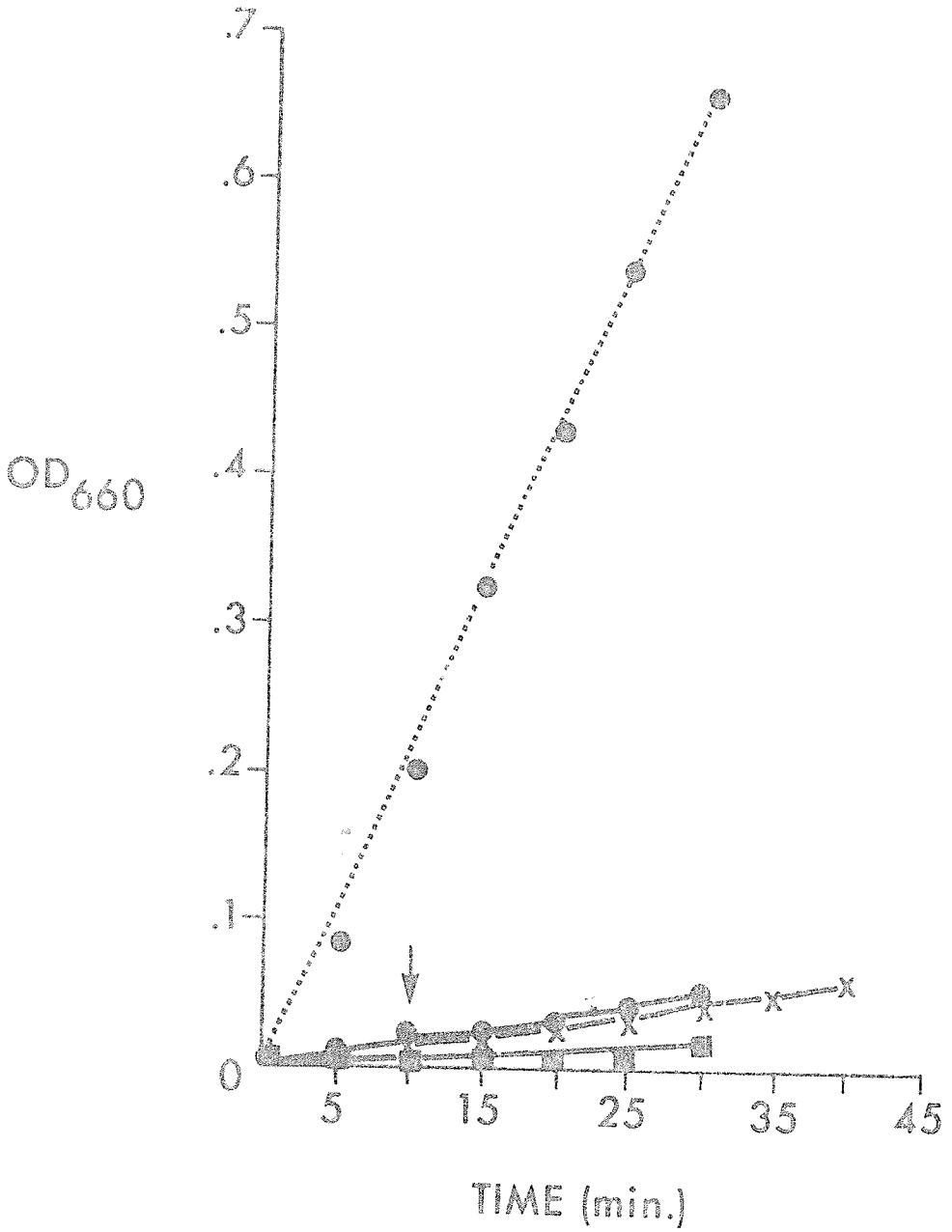


Figure 15: Test for the ability of actin to reverse the effects of the CMBPs on PDE using the PDE assay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.3
.2
.1
0
OD660
5
15
25
35
45
TIME (min.)

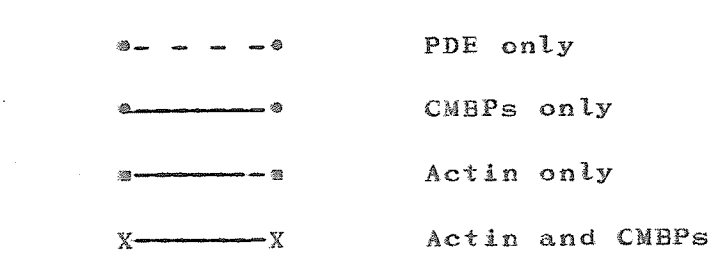
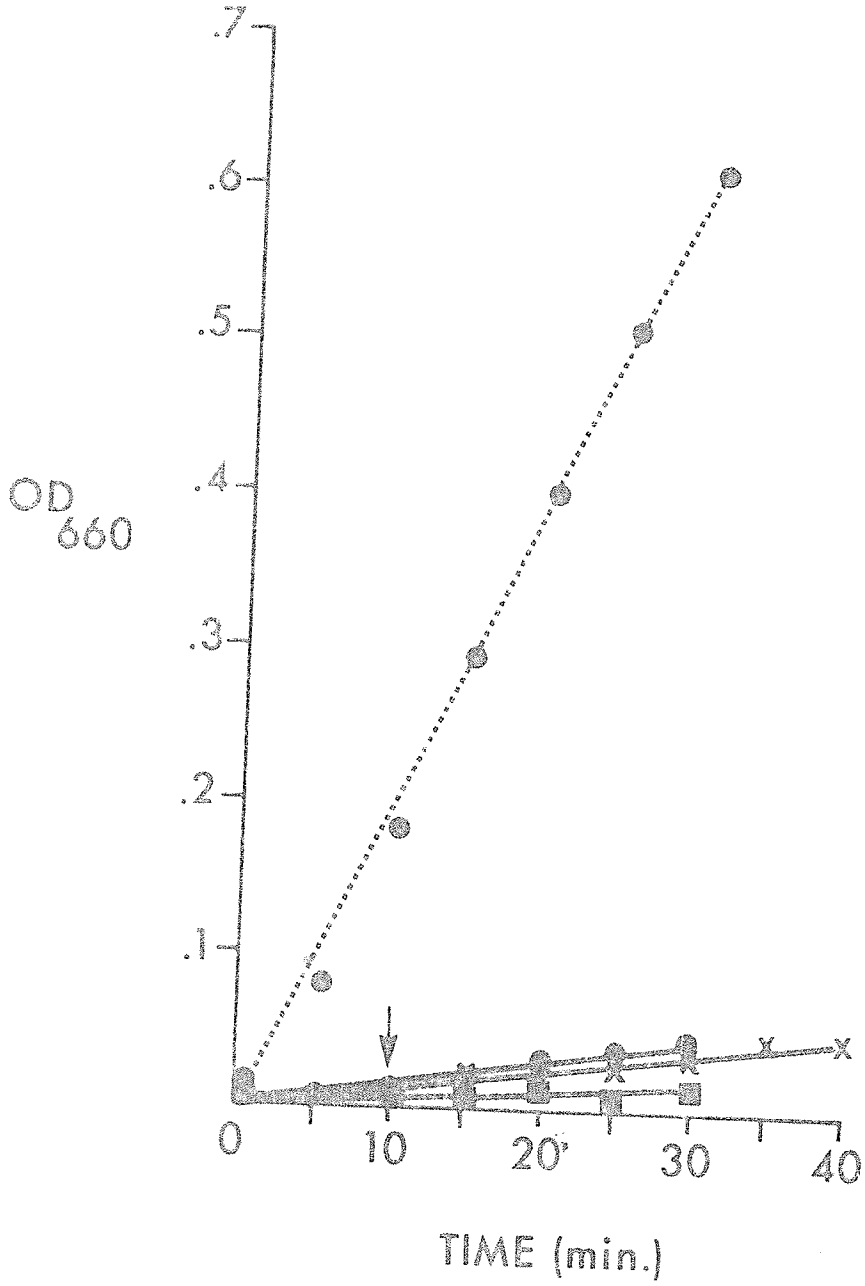


Figure 16: Test for the ability of actin to reverse the effects of the CMBPs on PDE using the PDE assay


.7
.6
.5
.4
.3
.2
.1
OD 660
0
10
20
30
40
TIME (min.)

Chapter V

DISCUSSION

There were two basic types of experiments performed in the present thesis to test the effect of CM and CMBPs on actin polymerization. The first type of experiment was to test whether there was an increase or decrease in the final steady state polymerization of actin as detected by viscometry. During these studies, actin was incubated with the various Ca^{+2} binding proteins and under the conditons used (namely 2 minute or 2 hour incubation periods, overnight equilibration, measurement at about $25^{0}C$ (or in one case $4^{0}C$), and the presence or absence of Ca^{+2}), no effect of these proteins could be observed. Table 13 summarizes all the viscometry studies performed.

Although the viscometry measurements obtained suggest that there was no effect of CM or the CMBPs on actin polymerization, there are further experiments which could be performed in the future to confirm this hypothesis. One of the first facts to consider is that rabbit skeletal muscle actin was used instead of bovine brain actin to interact with the bovine brain CM and CMBPs. Although the actin was isolated from a different source than the CM or CMBPs, there were two reasons for using skeletal muscle as a source for

TABLE 13

Summary Table Illustrating the Effect of Ca^{+2} Binding Proteins on G-Actin Polymerization

Experiment	Actin:CM (molar ratio)	CMBP's (ul)	Actin:CMBP-I (molar ratio)	Ca^{+2}/ EGTA	Specific Viscosity n(sp)
Effect of CM on Actin Polymerization in the presence of Ca^{+2}	5:0	-	-	Ca^{+2}	1.04
	5:3	-	-	Ca^{+2}	1.01
	5:5	-	-	Ca^{+2}	1.03
	5:10	-	-	Ca^{+2}	.98
Effect of CM on Actin polymerization with and without Ca^{+2}	5:0	-	-	Ca^{+2}	.86
	5:3	-	-	Ca^{+2}	.88
	5:5	-	-	Ca^{+2}	.85
	5:0	-	-	EGTA	.87
	5:3	-	-	EGTA	.86
	5:5	-	-	EGTA	.83
Effect of CM on the G-Actin to F-Actin conversion with and without Ca^{+2}	5:0	-	-	Ca^{+2}	.85
	5:2	-	-	Ca^{+2}	.86
	5:5	-	-	Ca^{+2}	.79
	5:10	-	-	Ca^{+2}	.90
	5:0	-	-	EGTA	.85
	5:2	-	-	EGTA	.84
	5:5	-	-	EGTA	.76
	5:10	-	-	EGTA	.80

TABLE 13

Summary Table Illustrating the Effect of Ca^{+2} Binding Proteins on G-Actin Polymerization (cont.)

Experiment	Actin:CM (molar ratio)	CMBP's (ul)	Actin:CMBP-I (molar ratio)	Ca^{+2}/ EGTA	Specific Viscosity n(sp)
Effect of total CMBP's on G-Actin polymerization with Ca^{+2}	5:0	-	-	Ca^{+2}	.92
	5:0	.064	-	Ca^{+2}	.88
	5:0	.160	-	Ca^{+2}	.93
	5:0	.288	-	Ca^{+2}	.91
Effect of total CMBP's on the G-Actin to F-Actin conversion with and without CM in the presence of Ca^{+2}	5:0	-	-	Ca^{+2}	.83
	5:5	-	-	Ca^{+2}	.77
	5:0	.013	-	Ca^{+2}	.80
	5:2	.013	-	Ca^{+2}	.82
	5:0	.033	-	Ca^{+2}	.79
	5:5	.033	-	Ca^{+2}	.81
	5:0	.066	-	Ca^{+2}	.81
	5:10	.066	-	Ca^{+2}	.79
Effect of total CMPB's on the G-Actin to F-actin conversion with and without CM in the presence of EGTA	5:0	-	-	EGTA	.80
	5:5	-	-	EGTA	.78
	5:0	.013	-	EGTA	.74
	5:2	.013	-	EGTA	.76
	5:0	.033	-	EGTA	.79
	5:5	.033	-	EGTA	.72
	5:0	.066	-	EGTA	.70
	5:10	.066	-	EGTA	.73

TABLE 13

Summary Table Illustrating the Effect of Ca^{+2} Binding Proteins on G-Actin Polymerization (cont.)

Experiment	Actin:CM (molar ratio)	CMBP's (ul)	Actin:CMBP-I (molar ratio)	Ca^{+2}/ EGTA	Specific Viscosity n(sp)
Effect of CMBP-I on G-Actin polymerization with Ca^{+2} (at lower actin concentration i.e. .168mg/ml)	5:0	-	-	Ca^{+2}	.00
	5:0	-	5:0.500	Ca^{+2}	.02
	5:0	-	5:0.560	Ca^{+2}	.06
	5:0	-	5:0.625	Ca^{+2}	.03
	5:0	-	5:0.715	Ca^{+2}	.02
	5:0	-	5:0.835	Ca^{+2}	.01
	5:0	-	5:1.000	Ca^{+2}	.03
	5:0	-	5:0.715	EGTA	.08
Effect of CMBP-I on the G-Actin to F-actin conversion in the presence and absence of Ca^{+2}	5:0	-	0:0	Ca^{+2}	1.19
	5:0	-	5:0.04	Ca^{+2}	1.08
	5:0	-	5:0.04	Ca^{+2}	1.16
	5:0	-	5:0.20	Ca^{+2}	1.04
	5:0	-	5:0.00	EGTA	1.13
	5:0	-	5:0.04	EGTA	1.21
	5:0	-	5:0.10	EGTA	1.20
	5:0	-	5:0.20	EGTA	1.19

TABLE 13

Summary Table Illustrating the Effect of Ca^{+2} Binding Proteins on G-Actin Polymerization (cont.)

Experiment	Actin:CM (molar ratio)	CMBP's (ul)	Actin:CMBP-I (molar ratio)	Ca^{+2}/ EGTA	Specific Viscosity n(sp)
Effect of CMBP-I on the G-actin to F-actin conversion with and without CM in the presence of Ca^{+2}	5:0	-	5:0	Ca^{+2}	.87
	5:0	-	5:0.04	Ca^{+2}	.83
	5:2	-	5:0.04	Ca^{+2}	.84
	5:0	-	5:0.10	Ca^{+2}	.93
	5:5	-	5:0.10	Ca^{+2}	.93
	5:0	-	5:0.20	Ca^{+2}	.80
	5:10	-	5:0.20	Ca^{+2}	.87
Effect of CMBP-I on the G-actin to F-actin conversion with and without CM in the presence of EGTA	5:0	-	0:0	EGTA	.93
	5:0	-	5:0.04	EGTA	.90
	5:2	-	5:0.04	EGTA	.93
	5:0	-	5:0.10	EGTA	.90
	5:5	-	5:0.10	EGTA	.92
	5:0	-	5:0.20	EGTA	.90
	5:10	-	5:0.20	EGTA	.89

actin. First, large amounts of actin could be extracted from rabbit skeletal muscle and this actin was highly purified (Huxley 1976). Secondly, the actin from various non-muscle sources has been shown to have many similar properties with skeletal muscle actin (Korn 1978). Skeletal muscle actin and non-muscle actin both have molecular weights of 42000 daltons as based on electrophoretic mobility on dodecyl sulfate/polyacrylamide gels. All actins contain 1 mole of bound adenine nucleotide per mole of protein and the amino acid composition of all the proteins is similar including the presence of one residue of $N^{3\beta}$-methylhistidine which is thought to be funtionally important. Further, similarity in amino acid sequences can be stressed by noting that in actins as evolutionarily different as Acanthamoeba castellanii and rabbit skeletal muscle, only about 6% of the residues are different (Korn 1978). There are also some differences between skeletal and non-muscle actins. The skeletal and heart muscle actins contain valine at positions 129, alanine at position 271, and tyrosine at position 278 while bovine brain and human platelet actins contain threonine, cysteine, and phenylalanine respectively at these positions. A. castellanii was found to contain some residues of muscle and non-muscle actins at these positions. At other locations in the molecule, A. castellanii actin differs in its residue composition from both types of vertebrate actin (Korn 1978).

Although A. castellanii and skeletal muscle actin do contain different residues, it is not known to what extent these differences contribute to polymerization since the proteins from both sources have been found to form random copolymers (Gordon, et al 1976; Eisenberg unpublished observations). It has been noted that the polymerization process of various non-muscle actins is qualitatively similar to skeletal muscle actin in terms of requirements for a critical concentration before nucleation and elongation can take place (Korn 1978). However, there are differences in the values of the critical concentration of non-muscle actins compared to skeletal muscle actins under non-physiological conditions (i.e. in the absence of Mg^{+2} or with KCl concentrations > 0.1M). Under these non-physiological conditions, the non-muscle actins tend to have higher critical conditions. However, it is not known how significant these findings are. If 2mM $MgCl_2$ is used, as probably exists in the cytosol, there is no significant difference in the critical concentrations of actin from various sources. For further experimentation, it would be interesting to note the effects of the Ca^{+2} binding proteins on bovine brain actin polymerization.

One could also investigate the effects of the CMBPs and CM on actin polymerization using a lower concentration of F-actin. In all studies (except one), 0.84 mg/ml of actin was used. This concentration of actin flowed through the

viscometer for a reasonable length of time, allowed for a detectable decrease in viscosity as would occur if depolymerization was occurring, and allowed for an increase in viscosity without increasing the flow-through time by too great a factor. If the flow-through times for each condition were too large, the final conditions would be equilibrating much longer that the initial conditions leading to an inconsistency in the experiment. However, in one study (Table 9), an actin concentration of 0.168 mg/ml was used and no effect of CMBPs on viscosity was observed.

Lastly, in relation to the viscosity studies, we were only interested in the final steady state value of actin polymerization since these are the only values which can be accurately measured by this technique. It would be interesting to note the effects of CM and the CMBPs on the initial stages of the actin polymerization reaction. This could be accomplished by using absorbance difference spectroscopy since as actin polymerizes, a conformational change occurs which leads to a change in its absorption spectrum. The maximum change occurs at 232nm (Higashve and Cosava, 1965; Spudich and Cooke, 1975). In these studies, the possiblily of an increase in G-actin polymerization in the presence these proteins would be matched with a G-actin blank without the proteins. This technique has been outlined by Gordon, et al (1976) for following the effects of various ions on actin polymerization.

The second type of study used was ultracentrifugation to show the effects of CM and/or the CMPBs, in the presence and absence of Ca^{+2}, on the polymerization rate of actin. Again, no effect of the Ca^{+2} binding proteins on this process was found. If the binding proteins were depolymerizing the F-actin, there should have been increasing amounts of actin found in the supernatants with increasing amounts of CM or CMBPs added. This was not observed. Furthermore, if the Ca^{+2} binding proteins were associated with the F-actin, they would have been found in the F-actin pellets. Again, this did not occur. Often, when proteins bind to F-actin, they are involved in gelation. Apart from not being found associated with F-actin as proteins involved in gelation would be, the solutions of actin, with other proteins, or actin alone, in the presence of EGTA or Ca^{+2}, did not gel when warmed to room temperature after overnight equilitration at 4^0C. In the past, gelation has been shown upon warming to 25^0C in the presence of EGTA (see section on the Ca^{+2} regulation of actin gelation). Based on these observations, it seems unlikely that these proteins are involved in the gelation process.

There are two other possibilities for future research. First, the ionic strength for $MgCl_2$ was physiological (2mM) and the concentration of KCl was slightly less that that considered to exist within the cell (50mM instead of 100mM). The pH for all studies was between 7.0 and 8.0. Based on

this, it is difficult to believe that ionic strength could inhibit the interaction betweer these proteins. However, Clark and Masters (1973) suggested that if the protein concentration of a solution is < 50.0 mg/ml (which is still less than some physiological protein concentrations) then some enzymes are unable to bind F-actin at physiological ionic strength and pH (see Enzymes that Bind Actin in the Litterature Review). Future research may investigate the effects of binding at higher protein concentrations. For example, bovine serum albumin could be included in the reaction mixtures at concentrations of 50.0 mg/ml to increase the protein concentration to near physiological values.

It could be questioned whether the protein concentration of the CMBP-I used could have been higher. However, it is difficult to conceive of higher concentrations being effective <u>in vivo</u> where only 52.0 mg of this protein is found per 1000.0g of bovine brain (Sharma, et al 1979). If 1000.0g of bovine brain were homogenized with 3.0 ml/g, then the final concentration of CMBP-I would be 0.017 mg/ml. On the other hand, when brain from embryonic chicks is homogenized at a similar volume/mass ratio, the final concentration of actin is 0.93 mg/ml (Gordon 1977). As an estimation, using these similar tissues, there would be approximately 55 times more actin (in adult brains this may be closer to 52) than CMBP-I on a weight basis. In these studies, there was between 64 and 13 times more actin than CMBP-I. This range of actin to

CMBP-I should have covered the possible ratios of these two proteins which may exist *in vivo*. When one considers that only 0.1 ug of pure CMBP-I is required to inhibit PDE, it seems unlikely that the concentrations used in this study are insufficient.

In conclusion, it appears that there was no effect of CM, with and without Ca^{+2}, on the steady state actin polymerization, depolymerization, or gelation. Although CM has been found in close proximity to actin *in vivo* (Means and Dedman 1980), it may be that this proximity is due to an association with other proteins which also bind actin. The CMBPs were not found to play such a role since they were not found to act in concert with CM on actin polymerization, nor did they reverse the effects of CM on this process. It seems, therefore, that a putative function for the CMBPs can not be found in their effect on the steady state actin polymerization.

Although CM has been found to regulate phenomena related to contraction, actin polymerization does not appear to be one such process. As outlined previously, CM has been found to enhance the ability of actin to activate the myosin ATPase via stimulation of the myosin light chain kinase which phosphorylates the myosin light chains. Unless actin is in its polymerized form, it is unable to perform this function. Therefore, it was questioned whether CM may be acting at the level of actin, as well as myosin, to regulate the AT-

Pase and therefore, contraction. The present results confirm the idea that CM regulation is associated with myosin rather than actin at least in relation to polymerization.

The similarity of CM to Tn-C also hinted at the suggestion that CM may bind to actin as does Tn in the presence of other components of the Tn-complex and Tm. However no evidence for this suggestion was obtained. Since Tn was found to regulate Ca^{+2} dependent phenomena assocated with skeletal muscle actin, it was questioned as to whether CM may be performing a similar function in tissues where Tn has not been found. In the present study, there was no effect of CM on the steady state actin polymerization with or without Ca^{+2}.

Although CM and/or the CMBPs were found to have no effect on actin polymerization, future experiments have been outlined to futher explore this contention.

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