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MILLS, JOHN STEVEN

INTERACTION OF CALCIUM, METAL IONS, AND CALMODULIN ANTAGONIST DRUGS AND TARGET PROTEINS WITH CALMODULIN

The Ohio State University PH.D. 1987

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INTERACTION OF CALCIUM, METAL IONS, AND CALMODULIN ANTAGONIST DRUGS AND TARGET PROTEINS WITH CALMODULIN

DISSertation

Presented in Partial Fulfillment of the the Requirements for the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By

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The Ohio State University

1987

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Advisor, Biophysics Program
To My Parents
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FIELDS OF STUDY

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LIST OF TABLES

Table I. Table of CaM affected proteins 9
Table II. Calmodulin antagonists 15
LIST OF FIGURES

Fig. 1. Correlation of the fluorescence increase observed in felodipine with its binding to CaM. 

Fig. 2. Scatchard analysis of fluorescence data of felodipine titrations of 1 μM calmodulin. 

Fig. 3. Hill plot analysis of fluorescence enhancement observed when 1 μM calmodulin is titrated with felodipine. 

Fig. 4. Hill plot analysis of fluorescence enhancement of felodipine titrated with calmodulin. 

Fig. 5. The fluorescence changes produced by titrations of calmodulin-felodipine with R24571, prenylamine and diltiazem are compared with a theoretical curve. 

Fig. 6. Scatchard analysis of felodipine binding to calmodulin in the presence of R24571 at various calcium concentrations. 

Fig 7. Calcium titrations of 2 μM calmodulin and 1 μM felodipine, felodipine and 10 μM prenylamine or calmodulin, felodipine, and 2 μM R24571. 

Fig. 8. Model for calmodulin interactions with calcium and drugs. 

Fig. 9. The effect of various metals on calmodulin-felodipine fluorescence. 

Fig. 10. The correlation between 3H felodipine binding and its fluorescence enhancement upon binding to felodipine. 

Fig. 11. Hill plots of felodipine binding to calmodulin alone and in the presence of zinc or in the presence of lanthanum. 

Fig. 12. Calmodulin titrations of felodipine alone and in the presence of lanthanum and in the presence of zinc. 

Fig. 13. Effect of calcium on the zinc mediated potentiation of calmodulin-felodipine fluorescence.
Fig. 14. The effect of zinc in potentiating calmodulin-felodipine fluorescence at different calcium and strontium concentrations.

Fig. 15. The effect of copper and mercury in reversing the highly fluorescent potentiated states produced by lanthanum and zinc.

Fig. 16. Effect of pH on calmodulin-felodipine fluorescence.

Fig. 17. Correlation between felodipine binding and terbium displacement from calmodulin.

Fig. 18. Reaction of MIANS with spinach and bovine brain calmodulin.

Fig. 19. Calcium titrations of MIANS labeled and native spinach calmodulin.

Fig. 20. Titrations of MIANS calmodulin with calcinurin, MLCK, and caldesmon.

Fig. 21. Scatchard analysis of MLCK and caldesmon binding to MIANS calmodulin.

Fig. 22. Activation of cGMP phosphodiesterase by unlabeled or MIANS labeled spinach calmodulin.

Fig. 23. Fluorescence emission spectra of DMSM, MIANS, acrylodan, or rhodamine X-maleimide labeled spinach calmodulin.
Introduction

It is now widely accepted that an increase in intracellular calcium ion concentration is universally employed to modulate and regulate many of the actions of eukaryotic cells. Excitable cells such as those in nerve and muscle use this calcium "signal" to initiate and coordinate events as important as neurotransmission and muscle contraction. Dividing cells use this calcium signal to facilitate a wide variety of biochemical events which eventually lead to replication and cell division. The cellular actions of many hormones, neurotransmitters, and drugs are mediated via an increase in cytosolic concentrations of this second messenger, calcium. It is critical that a cell maintain its calcium ion concentration at the appropriate level. In normal resting cells, extracellular calcium is as much as 10,000 fold in excess of intracellular free calcium. This calcium gradient is maintained by a variety of calcium channels, calcium antiporters, and calcium pumps located in the cell membrane, reticular membranes and mitochondrial membranes.

All eukaryotic cells react to stimuli (hormones, membrane depolarization, chemicals, antibodies, allergens, etc.) by increasing their cytosolic calcium levels. This calcium flux is mediated by a release of calcium from its intracellular stores (reticulum and/or mitochondria) or by the opening of calcium channels in the cell membrane which allow extracellular calcium to flow down its concentration gradient into the cell. As cytosolic calcium rises
into the micromolar range, it binds to calcium receptors which are generally referred to as calcium binding proteins. These intracellular calcium binding proteins are thereby activated and interact with their target protein within the cell to stimulate their enzymatic or biological activities. Many cells have specialized calcium binding proteins to regulate their cellular function in a calcium dependent manner. Perhaps the best example of this is the calcium binding protein troponin C. It is found in skeletal and cardiac muscle where it serves the specialized function of recognizing an increase in intracellular calcium and transducing this signal into actomyosin ATPase activity and muscle contraction (see 1 for review). Like troponin C, many of the calcium binding proteins discovered thus far serve a highly specialized function which is restricted to a limited number of tissues.

Calmodulin has emerged as the only calcium binding protein which can be regarded as a "universal" regulator of the calcium signal. Calmodulin is present in all eukaryotic cells. Its structure has been highly conserved throughout evolution and it is truely a multifunctional regulator of the calcium signal. Since its discovery by Cheung (2) and Kakiuchi and Yamasaki (3) in 1970 it has been shown to regulate more than thirty different proteins and enzymes in a calcium dependent manner. Some of these proteins, in turn, regulate many vital biological phenomena including neurosecretion, synthesis of neurotransmitter substances, glandular secretion, smooth muscle contraction, mitotic spindle formation, DNA replication, progression of the cell through the cell cycle, cell division, and cell differentiation. Thus, not only is calmodulin the only known
universal modulator of the calcium signal, it is also essential to all of the above vital cellular processes. Several recent reviews on calmodulin (4-9) and other calcium binding proteins (1,10,11) have appeared.

I. Distribution and Conservation of Structure of Calmodulin.

Although calmodulin is present in all eukaryotic organisms, its level in the cell varies from tissue to tissue (see 7 for review). In tissues such as brain, eel electric organ, and germ cells, calmodulin constitutes as much as 10% of the total protein (7). Even muscle tissues which abound with other more specific calcium binding proteins (including troponin and parvalbumin) have moderately high levels of calmodulin (40-50mg/kg of wet tissue). Intracellularly, calmodulin exists mostly in the cytosol but it has also been found in association with all cellular organelles except mitochondria where results are conflicting (12). Since calmodulin binds its target proteins in a calcium dependent manner, most cellular calmodulin can be released into the soluble fraction after homogenization in the presence of calcium chelators including EGTA and EDTA. Thus, calmodulin is a water soluble cytosolic protein which is associated with essentially all organelles in all eukaryotic organisms.

The primary structure of calmodulin has been highly conserved throughout evolution. This suggests, at least teleologically, that it
is involved in the regulation of processes which are fundamental to life and most lifeforms. Comparison of its primary amino acid sequence from several vertebrates, invertebrates, plants, and protozoans reveal this high degree of conservation (see 7 and 13 for review). It contains 148 amino acids and has a molecular weight of 16,500.

Structurally, calmodulin belongs to the family of troponin C-like or E-F hand proteins as defined by Kretsinger (10). Members of this family include cardiac and skeletal troponin C, parvalbumin, vitamin D dependent calcium binding protein, and S-100 protein. Of these proteins, only calmodulin has four functional calcium specific sites and it appears to be most closely related to their common ancestor protein (14). This ancestor calcium binding protein was composed of a pair of tandem calcium binding domains (E-F hands), quite similar to the structure of calmodulin. Thus, calmodulin may be the most ancient of this ancient family of calcium dependent modulators. Calmodulin appears to be unique among this family of proteins in that its cellular function is less specialized and it can interact and regulate multiple target proteins in a calcium dependent manner.

II. Structural Studies of Calmodulin

Calmodulin, like most calcium binding proteins, undergoes large structural changes as it binds calcium. Since it has four calcium binding sites it was predicted to have four E-F hands, as defined by Kretsinger (see 10). These E-F hands consist of a calcium binding loop (of 12 amino acids) flanked by two regions of nearly
perpendicular α-helix. With calcium binding, large increases in calmodulin's intrinsic tyrosine fluorescence (15), changes in its protein NMR spectra (22), U.V. absorption spectra (23), and an increase in α-helical content (15) occur. In addition, calcium binding exposes or forms hydrophobic regions on the surface of calmodulin. This has been observed by the calcium dependent binding of hydrophobic molecules, including the fluorescence probe molecules anilinonapthalenesulfonate and toluidynapthalenesulfonate, the calcium channel blocker felodipine, and many calmodulin antagonist drugs (24-27). The calcium dependence of the binding of these hydrophobic molecules parallels the calcium dependence of calmodulin's activation of many of its target proteins. Half-maximal activation occurs near pCa 6.0-6.1 and often exhibits a Hill coefficient of 3.0 (5,24,27,28). Many calmodulin antagonists drugs bind in a similar calcium dependent manner and prevent calmodulin's interaction with and activation of its target proteins. For these reasons, the calcium dependent hydrophobic sites are thought to be the regions where calmodulin interfaces with the proteins that it activates.

Many of these solution studies have now been confirmed by the recent elucidation of the three dimensional crystal structure of calmodulin to 3.0 Å resolution by Babu et al., (29). Calmodulin is a dumbbell-shaped molecule with two lobes connected by a long stand of helix (eight turns). The molecule is approximately 65 Å in length and each of the two non-interacting lobes is roughly 25x20x20 Å. The upper lobe contains calcium binding sites I and II (2 E-F hands) and the lower lobe calcium binding sites III and IV (2 E-F hands) and the protein is 63% α-helix.
Perhaps the most interesting feature is the long region of α-helix connecting calcium binding domains II and III and the separate halves of the molecule. Babu et al., (29) have proposed that this central helix may be buried in the absence of calcium and exposed upon calcium binding. This central helix appears to be important in facilitating calmodulin's calcium dependent interaction with drugs and proteins. Trypsin can cleave between residues 77-78 only in the presence of calcium, carboxymethylation occurs at methionine 71, 72 and 76 to inhibit calmodulin's interaction with phosphodiesterase and phenothiazine derivatives can be selectively attached to lysine 75 (see 29 for review). Thus, this central helix appears to be an important region for calmodulin's interaction with drugs and with the proteins it activates in a calcium dependent manner.

III. Ca\textsuperscript{2+} Binding to Calmodulin

Early studies of calcium binding to calmodulin showed 4 equivalent sites with a Kd=2.5 uM in the presence of EGTA buffers at pH 6.5, with 100 mM KCl. Mg\textsuperscript{2+} did not affect affinity (30). Following elucidation of the primary sequence by Watterson et al.(31) four putative Ca\textsuperscript{2+} binding loops (numbered I-IV from N-terminus) were predicted on the criteria of Kretsinger(10). However, recently the equivalency of the calcium binding sites has been challenged. Using a variety of methods, including 1H, 43Ca, and 113Cd NMR, [for a review see Forsen et al.(32)] authors have concluded that domains III and IV, both of which contain tyrosine in vertebrate calmodulin, have lower dissociation constants than do sites I and II. Stop flow
studies using the fluorescent chelator Quin 2 show that calmodulin exhibits two distinct calcium off rates, differing by a factor of ten (33). If the on rates are diffusion limited, and this may not be valid based on recent findings with Troponin C (34), then the calcium dissociation constants of the two domain pairs should also differ by a factor of ten. The fast and slow off rates were associated with the n and c terminal halves of calmodulin, respectively, based on similar rates with n and c terminal proteolytic fragments of calmodulin. Knowing how calcium interacts with calmodulin alone does not, unfortunately, allow one to determine directly to what extent a given calcium concentration will produce activation in vivo. This situation arises due to free energy coupling which allows some of the free energy decrease which occurs when \((\text{Ca}^{++})_{\text{calmodulin}}\) binds to a target protein, to be used to decrease the calcium dissociation constant for calmodulin. Thus at calcium concentrations well below those necessary to saturate free calmodulin (about 100 uM), complete activation of target proteins can occur, if the concentration of the target proteins in vivo are well above their dissociation constants for fully saturated calmodulin. This is usually the case. This free energy coupling process has been extensively evaluated in vitro for skeletal muscle light chain kinase-calmodulin interactions by Olwin et al. (35) and for phosphorylase kinase (Phos-K) by Burger et al. (36). Burger concluded that the binding of calcium to three of the four calcium domains in the calmodulin-Phos K complex was sufficient to allow activation for phosphorylase kinase, while the binding of four
calcium to calmodulin-myosin light chain kinase complex appeared to be necessary for its activation. Thus, the understanding of the energy linked process in individual cases is probably necessary before extrapolation to in vivo conditions can be made. 

IV. Processes Controlled by Calmodulin

At present, thirty or more proteins have been suggested as targets for calmodulin. Table 1 shows a list of some of the calmodulin target proteins which have been well characterized. In several instances, calmodulin is associated with the activation as well as the inactivation of the process. This is true for cAMP metabolism where both adenylate cyclase and phosphodiesterase are regulated by calmodulin. However, adenylate cyclase is not stimulated by calmodulin in all tissues (for review see (37) ) and calmodulin dependent phosphodiesterase catalyzes the hydrolysis of both cAMP and cGMP. Since cAMP and cGMP quite often have opposite effects on the cell, the effects of phosphodiesterase activation on the cell can not be determined a priori. The Na+-Ca++ antiporter is activated by a calmodulin dependent kinase and inactivated by a calmodulin dependent phosphatase (53). The result is not a futile cycle but a biphasic activation and inactivation curve, since the kinase is activated at low Ca++ concentrations and the phosphatase at higher Ca++ concentrations. Another example where calmodulin may modulate both activation and inactivation is myosin light chain phosphorylation. Myosin light chain kinase phosphorylates myosin light chains and phosphorylated myosin light chains can be dephosphorylated by calmodulin dependent phosphatase 2B. However, the major phosphatase in smooth muscle is a calmodulin independent
Table 1

Table of CAM Affected Proteins

<table>
<thead>
<tr>
<th>Cyclic Nucleotide Metabolism</th>
<th>K0.5 CAM</th>
<th>PK0.5 Ca++ Activation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenylate Cyclase</td>
<td>10^{-9}</td>
<td>6.7-6.9</td>
<td>5</td>
</tr>
<tr>
<td>Guanylate Cyclase</td>
<td>ND</td>
<td>5.0</td>
<td>8</td>
</tr>
<tr>
<td>Cyclic Nucleotide Phosphodiesterase</td>
<td>10^{-9}</td>
<td>5.5-6.0</td>
<td>20</td>
</tr>
</tbody>
</table>

Calmodulin Dependent
Protein Kinases and Phosphatases

<table>
<thead>
<tr>
<th>Kinase</th>
<th>K0.5 CAM</th>
<th>PK0.5 Ca++ Activation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multifunctional CAM dependent kinase</td>
<td>1.2 x 10^{-8}</td>
<td>5.7</td>
<td>200</td>
</tr>
<tr>
<td>Myosin light chain kinase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smooth muscle unphosphorylated</td>
<td>4 x 10^{-9}</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Smooth muscle phosphorylated</td>
<td>5 x 10^{-8}</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>6 x 10^{-8}</td>
<td>&gt;20</td>
<td>28</td>
</tr>
<tr>
<td>PP57 kinase</td>
<td>4 x 10^{-8}</td>
<td>ND</td>
<td>&gt;20</td>
</tr>
<tr>
<td>Caldesmon kinase</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Phosphorylase kinase</td>
<td>4 x 10^{-8}</td>
<td>7.0</td>
<td>6</td>
</tr>
<tr>
<td>Ornithine decarboxylase kinase</td>
<td>ND</td>
<td>ND</td>
<td>7</td>
</tr>
<tr>
<td>Tyrosine hydroxylase kinase (or Tryptophan hydroxylase kinase)</td>
<td>10^{-8}</td>
<td>5.9</td>
<td>33</td>
</tr>
<tr>
<td>Phospholamban kinase</td>
<td>10^{-8}-10^{-9}</td>
<td>5.5-6.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Protein phosphatase (calcineurin)</td>
<td>10^{-8}</td>
<td>6.2</td>
<td>20</td>
</tr>
</tbody>
</table>

Proteins involved in ion fluxes directly or indirectly regulated by calmodulin

1. Na^+ K^+ ATPase 10^{-7} ND 0.4 52
2. Na^+ Ca^{2+} Antiporter Kinase ND 6.0 NA 53
3. Na^+Ca^{2+} Antiporter Phosphatase ND 5.5 NA 53
4. Ca^{2+} Mg^{2+} ATPases (Plasma membrane) 10^{-7}-10^{-9} 5.5 3.5 54

Other Proteins Which Bind to Calmodulin

<table>
<thead>
<tr>
<th>Protein</th>
<th>K0.5 CAM</th>
<th>PK0.5 Ca++ Activation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troponin I</td>
<td>2 x 10^{-8}</td>
<td>5.8</td>
<td>NA</td>
</tr>
<tr>
<td>Phospholamban</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Glycogen phosphorlyase b</td>
<td>ND</td>
<td>ND</td>
<td>1.0</td>
</tr>
<tr>
<td>Dynein ATPase</td>
<td>10^{-7}</td>
<td>&lt;5</td>
<td>8</td>
</tr>
<tr>
<td>Spectrin (fodrin)</td>
<td>&lt; 3 μM</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>NAD Kinase</td>
<td>3 x 10^{-11}</td>
<td>ND</td>
<td>100</td>
</tr>
<tr>
<td>Caldesmon</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Calmodulin binding glycoprotein</td>
<td>2 x 10^{-8}</td>
<td>ND</td>
<td>NA</td>
</tr>
<tr>
<td>Heat Stable Phosphodiesterase Inhibitor</td>
<td>&lt; 10^{-9}</td>
<td>ND</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA = Not applicable
ND = Not determined
phosphatase (64) so that in smooth muscle calmodulin may not regulate dephosphorylation. Therefore, in those cases where calmodulin activation may appear to produce a futile cycle is is (1) important to determine whether both processes occur in the tissue, (2) the calcium dependence of each process, and (3) whether the product of the first process is the only substrate or enzyme utilized by the second process. In the case of enzymes, the usual criteria for a calmodulin dependent process has been an effect on enzyme activity. This has clearly been shown for cyclic nucleotide phosphodiesterase, myosin light chain kinase, phospholamban kinase, and multifunctional calmodulin dependent protein kinase where the enzyme activity is absolutely dependent on calmodulin (stimulation >50 fold; most of these enzymes can be rendered independent of calmodulin by proteolysis). However, in cases like phosphorylase kinase (36) and calcineurin (50,51), which contain intrinsic calcium binding proteins, stimulation by calmodulin is more modest. In other cases like binding to phosphorylase b (59), (calmodulin does not bind to phosphorylase a), the binding produces no detectable affect on activity and phosphorylase b cannot inhibit activation of cyclic nucleotide phosphodiesterase by calmodulin. In this case, the authors have suggested that the binding may not be physiologically relevant. Thus, activation of an enzyme or inhibition of a known calmodulin dependent process should be a minimum criteria of relevant calmodulin binding.

In the case of the calmodulin binding glycoprotein bound in macrophages, physiological relevance was shown by producing cell line variants which lacked this calmodulin binding protein, thereby showing
that this protein was important in regulation of growth (62). Such an approach is useful when the calmodulin binding protein has no known enzymatic activity.

In several cases, binding of calmodulin to target proteins has been shown to be reduced when these target proteins are phosphorylated (42,65-67). In the case of myosin light chain kinase, the binding can be altered by phosphorylation by either cAMP dependent protein kinase or protein kinase C, (a Ca++, but not calmodulin, regulated protein kinase). Thus, it can be expected that the regulation of myosin light chain kinase by Ca++ is rather complex, and is not solely dependent upon calmodulin. In the case of calcineurin, activation by calmodulin is time dependent and of short duration (68). This indicates that activation in vivo may occur only for short periods even if the concentration of Ca++ is not reduced, thereby allowing different target proteins to be activated on a different time scale.

V. Regulation by Ca++

In several systems, activation by calmodulin is modulated by direct interactions of Ca++ with the target protein. Adenyl cyclase is directly inactivated by Ca++ but stimulated by Ca++-calmodulin (39). This results in a biphasic Ca++ response curve with maximal activity at 0.2μM Ca++ (EGTA Buffer). Similar curves are seen with guanylate cyclase (39). Thus, these enzymes show maximal activity at low Ca++ concentrations. Ca++-Mg++ ATPase is dependent on Ca++ for calmodulin activation since calmodulin binds to the Ca++-Enzyme with a 20 fold higher affinity than to the enzyme alone.
Therefore at low Ca++, calmodulin binds poorly to Ca++ Mg++ ATPase whereas it binds tightly at high Ca++ concentrations, the result being that Ca++-Mg++ATPase is activated at higher calcium concentrations than most other calmodulin target proteins. It is tempting to speculate that adenylate cyclase at high Ca++ may exhibit low affinity for calmodulin in an exactly opposite manner to that proposed for calmodulin binding to Ca++ Mg++ ATPase i.e. high affinity for Ca++ free adenyl cyclase and lower affinity for Ca++ containing adenyl cyclase. Cyclase containing membranes exhibit both high and low affinity calmodulin binding sites, although it is not known whether the low affinity sites are binding to Ca++-adenyl cyclase. It will be necessary to purify cyclase to homogeneity before such questions can be answered.

In some systems Ca++ may modulate the target protein's response to calmodulin in a complex manner. As been previously stated, the Na⁺ Ca++ antiporter is a substrate of both calmodulin dependent protein kinase and phosphatase, each with a different Ca++ sensitivity. This allows the antiporter to exhibit maximal activity at a set Ca++ concentration. This may presumably be the case for many of the target proteins which are activated by calmodulin dependent kinases, since the major calmodulin dependent phosphatase calcinurin has been reported to have a rather broad substrate specificity.

VI. Effects of other cations besides calcium on calmodulin

Although calmodulin is generally considered to be only a calcium binding protein, there is increasing evidence that the activity of
calmodulin may also be affected by other metal ions. Brewer et al. (69,70) have proposed that zinc can antagonize the actions of calcium via interactions with calmodulin. Zinc was shown to inhibit the calmodulin dependent activation of Ca++ Mg++ ATPase of erythrocytes with half maximal effects at 40μM zinc. Similar inhibitory effects were observed on the activation of cyclic nucleotide phosphodiesterase [PDE](71). PDE inhibition by zinc was non-competitive (Ki 70μm at 5 mM Mg++) with both Ca++ and calmodulin, implying that zinc probably binds to calmodulin in the presence of Ca++ to produce an inhibitory form of calmodulin. However, in the case of Ca++-Mg++ ATPase, zinc may interact directly with the calcium binding site on Ca++-Mg++-ATPase since zinc also affects basal enzyme activity.

Cox and Harrison (72) have shown that a good correlation exists between the ability of a metal to inhibit Ca++ dependent activation of phosphodiesterase and the toxicity (LD 50) of metals in vivo. This suggests that interactions between metals and calmodulin produce important physiological effects and may result in metal toxicity.

Recently, the crystalline structure of calmodulin has been elucidated using Pb++ and Pt++ as heavy atom derivatives of calmodulin. Pb++ displaced Ca++ from calcium binding domains 1 and 2 in the crystalline structure(29). Cd++ and Tb++ have also been shown to bind to these domains with high affinity (32,73). Both Pb++ and Pt++ have been shown to inhibit the activation of phosphodiesterase in the presence of Ca++(72).
VII. Drugs as Allosteric Regulators of Calmodulin

The number of drugs which have been shown to interact with calmodulin is enormous. [See Table II for a sample] Recently, Van Belle has reported that of more than 5000 drugs screened for binding to calmodulin almost 20% interacted with calmodulin with an affinity equal to the widely used calmodulin inhibitor trifluoperazine. Very few drugs are selective inhibitors of calmodulin. Many neuroleptic drugs interact with calmodulin but most bind to dopamine receptors with at least 100 fold higher affinity than to calmodulin. Even DDT, a well known pesticide, has been reported to be a very potent inhibitor of calmodulin. At present, there are two compounds which seem to exhibit some selectivity for calmodulin. One is R24571 (calmidazolium) a derivative of an anti-mycotic drug and the other is compound 48/80 (74), a mixture of amphilic cations which are condensation products of N-methyl-phenethylamine and formaldehyde. Neither compound is likely to be membrane permeable and their use is therefore largely restricted to in vitro inhibition.

Calmodulin is known to expose hydrophobic regions on its surface upon binding of Ca++. It is these hydrophobic regions to which many drugs are presumed to bind (24-26). Most drugs are cationic amphiles but fluorescent anionic amphiles like TNS and ANS also bind. In addition non charged compounds like felodipine, diltiazem and verapamil also bind to calmodulin. Some compounds are capable of acting as suicide inhibitors. An example is ophiobolin A, a fungal metabolite and phytotoxin (90).
## TABLE 2

**CALMODULIN ANTAGONISTS**

<table>
<thead>
<tr>
<th>Drugs</th>
<th>Kd PDE</th>
<th>Kd Binding</th>
<th>Moles/mol</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>R24571</td>
<td>.005</td>
<td>.003</td>
<td>ND</td>
<td>74-77</td>
</tr>
<tr>
<td>Compound 48/80</td>
<td>.015</td>
<td>&lt;.050</td>
<td>ND</td>
<td>74</td>
</tr>
<tr>
<td>Vitamin D (25-OH-D)</td>
<td>.26</td>
<td>.03</td>
<td>ND</td>
<td>78-79</td>
</tr>
<tr>
<td>Pimozide</td>
<td>0.7</td>
<td>.83</td>
<td>1</td>
<td>80,81</td>
</tr>
<tr>
<td>Penfluridol</td>
<td>.47</td>
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Tanaka and Hadaka (26) have observed some selectivity with respect to drugs ability to inhibit various calmodulin target proteins. Prenylamine was 3.5 times more effective than W-7 in inhibiting cyclic nucleotide phosphodiesterase (PDE) but less effective than W-7 at inhibiting Ca\textsuperscript{++} Mg\textsuperscript{++} ATPase. In addition, there was no correlation between drug concentrations which produced 50% inhibition of PDE, myosin light chain kinase, Ca\textsuperscript{++} Mg\textsuperscript{++} ATPase and drug concentrations which produced 50% inhibition of W-7 binding, implying that either drug inhibition occurs via allosteric mechanisms or that different drugs have different binding sites on calmodulin or both. It appears that it may be possible to tailor drugs to inhibit (or activate) calmodulin proteins selectively. To do this effectively, it will be necessary to determine the interactions between drugs and calmodulin quantitatively, to determine the calmodulin conformations to which the drugs bind, and to design drugs which do not interact with hydrophobic binding sites on other proteins.

VIII. Cooperative interactions between CaM and PDE.

Recently, Cox et al. (40) has shown that cyclic nucleotide phosphodiesterase interacts with calmodulin in a cooperative manner. This was shown to be the case for either CaMcalmodulin or SrMcalmodulin where Hill coefficients of activation by the active calmodulin species were shown to be 1.6 and 2.0 respectively with K\textsubscript{0.5} of 0.8 and 1.2 nMolar. Several authors have shown that the activation by calmodulin is hyperbolic (ie no cooperactivity) but the difference appears to be due to the condition of the PDE assay. Many authors do their phosphodiesterase assay at pH 8.0 or in the presence
of imidazole which has been shown to stimulate phosphodiesterase activity. Kachuk and Menshikov (95) have shown that stimulation of phosphodiesterase is very dependent upon pH with greater activation and a greater degree of cooperativity occurring at pH 7.0 than pH 8.0. Moreover the degree of stimulation is variable. The relationship between the degree of stimulation and degree of proteolysis has been extensively investigated by Tucker et al. They show that even minor proteolysis can reduce the degree of stimulation from greater than 60 fold for "intact" enzyme to values of 6 or less with minor proteolysis. Gopalakishna and Anderson (97) have shown that the highly stimulatable form (> 60 fold) of PDE can be resolved from the more modestly stimulatable (6 fold) form on phenyl sepharose. Stimulation by calmodulin could be abolished by 50% ethylene glycol, indicating that hydrophobic interactions play a major role in the interactions between calmodulin and phosphodiesterase. These two methods allow PDE to be isolated in an almost completely calmodulin dependent form. This is necessary for accurate evaluation of calmodulin binding to PDE since the calmodulin regulation can be abolished by proteolysis.

Considerable evidence has been mounting indicating cooperative interactions between calmodulin and PDE. Derivatives of calmodulin (norchlorpromazine-calmodulin), as well as the calmodulin fragment 78-148 act as calmodulin antagonists but are devoid of ability to stimulate phosphodiesterase activity. These observations can be explained using a simple theoretical model. In this model, each dimer of PDE binds two calmodulin molecules [evidence for this species has been shown using crosslinking by Laporte et al.(98)]. Each dimer
contains two binding sites for calmodulin. Since calmodulin contains
two hydrophobic domains it probably binds to a hydrophobic site on
each PDE subunit and each subunit contains two hydrophobic sites. If,
in the active conformation, these hydrophobic domains on PDE are
situated such that they can interact with the two hydrophobic binding
domains on calmodulin but the hydrophobic domains in the inactive
conformation of PDE are not so situated, then calmodulin will
interact selectively with the active conformation and drive the
reaction toward this conformation. Binding of a second calmodulin
molecule provides additional binding energy to pull the reaction
toward active conformation. If one of the hydrophobic domains is
blocked by covalent interaction with a hydrophobic drug
(i.e. norchlorpromazine) then the derivatized calmodulin can no longer
interact selectively with the active conformation and the derivative
will act as an inhibitor. Blocking both hydrophobic binding sites on
calmodulin should completely abolish activity. This has been shown
using several calmodulin derivatives (4,99).

IX. Selective activation of calmodulin target proteins using
modified calmodulins. Attempts have been made to prepare
modified calmodulins which can activate selectively some target
proteins. Originally studies were carried out using the
n-terminal and c-terminal proteolytic fragments of calmodulin
(4). Both fragments could activate phosphorylase kinase, but no
other enzyme tested. The c-terminal fragment acted as
antagonist to both cyclic nucleotide phosphodiesterase (PDE) and
myosin light chain kinase (MLCK), but not to calcineurin or calmodulin dependent protein kinase (CaM-PK). Also a covalently modified calmodulin to which the calmodulin antagonist drug norchlorpromazine was attached to lysine 77 has been prepared (4). This derivative acts as antagonist to PDE and MLCK, a partial agonist toward calcinurin, and a full agonist toward CaM-PK and phosphorylase kinase. Recently, a chicken calmodulin pseudogene has been produced in bacteria using genetic engineering techniques (100,101). This calmodulin contains 16 amino acid changes throughout the 148 amino acid residues of calmodulin. It has a slightly reduced affinity for calcium, and has a three fold higher dissociation constant for PDE, with a marked reduction in the Hill coefficient for calcium activation (from n=5 to n=2). It has a dissociation constant for MLCK, however, that is one half that of native calmodulin. It acts as a partial agonist toward calcinurin and CaM-PK, and is essentially inactive in activating phosphorylase kinase. Studies with genetically engineered plant calmodulin which lacks the post translationally modified trimethyllysine have shown that the unmodified calmodulin acts a better agonist than does the modified form toward plant NAD+ kinase (61). Until recently, it had generally been assumed that all vertebrate and plant calmodulins were trimethylated. However, Rowe et al. (102) have isolated calmodulin N-methyl transferase and shown that some tissues, especially skeletal muscle, contain hypomethylated calmodulin. Thus it appears that even small changes in the primary structure of calmodulin can affect its activation of target proteins.
1. COOPERATIVITY AMONG CALMODULINS BINDING SITES

INTRODUCTION

Calmodulin is a ubiquitous calcium binding protein which binds four moles of calcium per mole of protein and undergoes large calcium dependent changes in structure. These structural changes form or expose hydrophobic binding sites on its surface (25,26) where calmodulin binds and activates as many as thirty different proteins in a calcium dependent manner (see 6 and 7 for review). Certain drugs, including trifluoperazine (TFP), W-7, and R24571, can bind to these sites with high to moderate affinity and inhibit calmodulin's interaction and activation of many of the proteins that it modulates. Presently, it is uncertain how calmodulin can bind and selectively activate so many different proteins with any degree of specificity.

Several models have been proposed to explain how calmodulin might exhibit selectivity as a calcium dependent modulator. For example, some target proteins appear to exhibit different $K_{0.5}$ for calmodulin (103). Further, the calmodulin species which activates the target may vary as a function of [Ca++]$.$ Halech et al. (20) have provided evidence for an ordered binding of calcium which might activate CaM for specific functions dependent upon the free Ca++ ion concentration. In addition, different proteins may interact with different binding sites on calmodulin allowing some degree of selective activation. All of the above mechanisms may contribute to the selectivity of CaM in activating its various target proteins.

Previously, we have shown that the dihydropyridine calcium
antagonist felodipine binds to calmodulin in a calcium dependent manner and that this binding (fluorescence) can be enhanced by the interaction of calmodulin antagonists at distinct sites via allosteric mechanisms (24). It has been suggested that these allosteric interactions among the drug/protein sites on calmodulin might provide a mechanism for selectively directing calmodulin to specific target proteins.

In this model, endogenous regulators (chemicals, peptides, or proteins) would bind to calmodulin and alter its conformation via these allosteric mechanisms. These various calmodulin conformers would have an altered protein binding site which would now exhibit enhanced selectivity for activating certain target proteins over others.

In an effort to more fully understand the mechanism of the allosteric interactions among the drug binding sites on calmodulin, we have analyzed the binding of felodipine to calmodulin in the presence and in the absence of its potentiating drugs. We propose a model to explain how cooperativity among calcium dependent drug/protein binding sites may work in concert with the cooperativity among the calcium binding sites to produce distinct conformers of calmodulin for selective activation of its target proteins.
**Materials and Methods**

**Equilibrium dialysis** - Buffer used was 10 mM Mops pH 7.0, 2 mM EGTA, 3 mM CaCl₂ and 90 mM KCl (Buffer A). ³H felodipine of constant specific activity was added to the solution (50 ml) outside the dialysis bag which contained 3 ml of 1 μM calmodulin. Specific activity was determined using a millimolar extinction coefficient at 370 nm of 6.4 in 95% ethanol.

**Fluorescence enhancement** - Fluorescence measurements were carried out in buffer A using 0.2 μM calmodulin and 1 μM felodipine and varying amounts of calmodulin antagonists. For titrations with felodipine (dissolved in ethanol), buffer A was used with ethanol held fixed at 4%. For felodipine titrations in the presence of prenylamine or R24571, the order of addition was felodipine, calmodulin, and then R24571 or prenylamine. Fluorescence measurements were carried out as previously described (24), free [Ca] was determined using an EGTA-Ca²⁺ buffer systems as previously described (104) and calmodulin concentrations were determined using E₂₇₈=3190. Hill plots were analyzed by an iterative linear regression Hill plot program and Bₘₐₓ was determined by the best fit of the data to the Hill equation.

Felodipine and ³H felodipine were gifts of A.B. Hässle Pharmaceutical, Mölndal, Sweden. R24571 was obtained from Janssen Laboratories. Prenylamine and diltiazem were obtained from Chinion and Marion Pharmaceuticals, respectively. All other chemicals were reagent grade. Calmodulin was purified from bovine testes as previously described (105).
Results

To determine whether the previously reported increase in felodipine fluorescence observed upon the addition of prenylamine to calmodulin-felodipine was due to enhanced felodipine binding or increased quantum yield of bound felodipine, equilibrium dialysis was carried out at varying concentrations of felodipine in the absence or presence of 10μM prenylamine (PA). This concentration of PA has previously been shown to produce optimal enhancement (24). A plot of ^3H felodipine binding vs the fluorescence difference between inside and outside of the dialysis bag (Figure 1), shows that the quantum yield is enhanced 5.6 fold when 1 mol of felodipine is bound per mol calmodulin, both in the presence and absence of prenylamine. The increase in fluorescence produced by prenylamine is, therefore, due to enhanced felodipine binding. Further, this curve shows the close correlation between felodipine binding and its fluorescence enhancement in both the presence and absence of potentiating drug and was subsequently used to quantitate felodipine binding.

A Scatchard plot of the fluorescent data obtained from felodipine titrations of calmodulin (B vs B/S^2) gave a straight line (indicating that the Hill coefficient was 2) with a B_{max} of 1.9 moles/mole and a K_{0.5} of 22 ± 4μM (r=0.90) (Figure 2.A). Scatchard analysis in the presence of 10μM prenylamine showed a reduction of both K_{0.5} (K_{0.5}=4μM) and B_{max} (B_{max}=0.8), whereas neither K_{0.5} or B_{max} was altered appreciably by 2μM R24571. Figure 3 shows Hill plots (B_{max}=1.9) of calmodulin titrated with felodipine alone and in the presence of 2μM R24571 or 10μM prenylamine,
concentrations which optimally enhance felodipine-calmodulin fluorescence. These Hill plots show that the fluorescence enhancement results from a reduction in the Hill coefficient from 2 to 1 for R24571 and from 2 to 0.6 for prenylamine.

In an effort to determine the effects of prenylamine and R24571 on the $K_{0.5}$ of calmodulin for felodipine, titrations of felodipine (± prenylamine or R24571) with calmodulin were carried out. Hill plots of these titrations are shown in Figure 4. Hill coefficients of 1.1 and 1.0 were obtained in the presence of R24571 and prenylamine, respectively, compared to 0.8 with felodipine alone. The data indicate that both prenylamine and R24571 decrease the apparent $K_{0.5}$ of calmodulin for felodipine from 14μM to 0.5μM and 0.6μM, respectively. Thus, the data from Fig 3 and Fig 4 indicate that prenylamine and R24571 reduce the Hill coefficient from 2 to 1 or less, and enhance the affinity of calmodulin for felodipine at the allosterically affected felodipine site by approximately 25-fold.

The fluorescence increases and decreases observed when felodipine-calmodulin is titrated with prenylamine, R24571 and diltiazem, are compared with a theoretical curve in Figure 5 (upper). The F curve (Δ--Δ) shows the fluorescent enhancement plot one would expect to see if one titrated calmodulin-felodipine (1μM) with a nonfluorescent analogue of felodipine which had the same $K_{0.5}$ as felodipine. This is represented by a plot of % felodipine bound minus % felodipine bound at 1μM (data taken from Fig. 2A and 100% fluorescence = 4% felodipine bound). Also shown is the theoretical (T) fluorescence enhancement one would expect from a titration of calmodulin-felodipine with a drug which has two equivalent sites on
calmodulin, assuming that felodipine binds only to those calmodulin molecules which have one and only one drug molecule bound. The plots of $\sum S \Delta F \cdot d(\log S)/\sum \Delta F \cdot d(\log S)$ vs log S, shown in Fig. 5 (lower), give the expected S shaped drug titration plots. Prenylamine is most similar to the theoretical two equivalent site model (Hill coefficient=0.8) indicating that felodipine binds with high affinity to those calmodulin molecules containing one and only one prenylamine, but that prenylamine does not potentiate its own binding. Both diltiazem and R24571 are similar to what one would expect for nonfluorescent analogues of felodipine and exhibit Hill coefficients near 2.0. These latter drugs potentiate binding of both felodipine and themselves to a second allosterically regulated site.

Therefore, there appear to be at least two classes of drugs that bind to calmodulin. Class 1: Those drugs that potentiate felodipine binding to a second site, but have similar affinities for both sites themselves, exhibiting Hill coefficients of 1 or less. Class 2: Drugs which exhibit positive cooperativity (Hill coefficient = 2) and potentiate binding of both themselves and felodipine to a second site.

This study illustrates another major difference between PA and R24571. Although PA, R24571, and diltiazem can each potentiate felodipine binding at pCa 3.0, at much lower [Ca++] only R24571 is an effective potentiator. Fig 6 (inset) shows the effect of R24571 on calmodulin-felodipine fluorescence at pCa 7.0. At pCa=7.0, the $K_{0.5}$ of R24571 for CaM-felodipine is estimated to be 1 µM and it again enhances CaM-felodipine fluorescence with a Hill coefficient of 2.0 (r=0.998). At pCa 7.0 neither prenylamine (at 100 µM), diltiazem (at 500 µM) nor felodipine itself (at 10µM) will potentiate felodipine
binding to calmodulin. Figure 6 shows Scatchard analysis of felodipine titrations of calmodulin in the presence of R24571 at pCa 7.0, 4.0 and 3.0. These data show a reduction in B max at lower Ca++ concentrations. This is consistent with a reduction in the number of active CaM molecules at lower [Ca++]]. No fluorescence enhancement was noted at pCa = 7 with felodipine alone or in the presence of 10μM prenylamine. Figure 7 shows the calcium dependence of the fluorescence increase produced by felodipine binding to calmodulin. This increase is Half-maximal at pCa = 6.0 with a Hill coefficient of 4. The addition of 2μM R24571 dramatically influences the calcium dependence of these fluorescence changes. In the presence of R24571 the calcium curve is clearly biphasic and felodipine binding is potentiated by calcium binding to sites with K0.5 estimated to be pCa 7.6 and pCa 6.0. The presence of 10μM PA has little effect on calcium dependence of felodipine-calmodulin but the Hill coefficient was reduced from 4 to 3. These data suggest that at pCa 7.0, R24571 but not 10μM prenylamine can potentiate felodipine binding to calmodulin and that, in the presence of felodipine, R24571 but not prenylamine can enhance the calcium affinity of some of the calcium binding sites on CaM.
Discussion

In this study we have explored the mechanism for allosteric drug effects on calmodulin-felodipine fluorescence in a quantitative manner.

We find that CaM has two binding sites for felodipine ($K_{0.5} = 22\mu$M) and that these sites exhibit strong positive cooperativity (Hill coefficient=2.0). Calmodulin antagonists (CaM-ANTs), such as prenylamine and R24571, enhance felodipine binding by decreasing the Hill coefficient for its binding from 2.0 to 0.6 and 1.0, respectively. These nonfluorescent CaM-ANTs presumably bind to one of the two felodipine binding sites and potentiate felodipine binding to the remaining felodipine binding site. Our titrations of felodipine with calmodulin in the presence of its potentiators (prenylamine and R24571) show that felodipine now binds to a single site whose dissociation constant has been reduced from $14\mu$M to $0.5\mu$M and $0.6\mu$M, respectively. Thus, prenylamine and R24571 enhance felodipine-calmodulin fluorescence by binding to a single site on calmodulin and allosterically enhancing the affinity of felodipine at a second binding site.

Felodipine binding and its potentiation by prenylamine and R24571 are strictly dependent on calcium. Under low Ca$^{++}$ conditions (pCa 10 - pCa 7.0) felodipine will not bind and prenylamine ($100\mu$M) will not potentiate its binding as it does at pCa 3.0 or pCa 4. R24571, however, can potentiate felodipine binding even at pCa 7.0. R24571 in
the presence of felodipine produces an apparent increase (40 fold) in the affinity of some of the calcium binding sites that regulate felodipine binding (Figure 7). This suggests that prenylamine and R24571 exhibit some fundamental differences in their binding and that only R24571 can enhance calcium binding to calmodulin such that felodipine will bind at pCa 7.0. The potentiation of felodipine binding at pCa 7.0 by R24571 may be due to its high affinity for calmodulin or may reflect some selectivity in its binding. Johnson and Wittenauer (77) have reported a K_d of 2-3nM for R24571 and dansylated calmodulin. Therefore, of the drugs tested, only R24571 can bind to calmodulin with high enough affinity to pull the reaction toward the calmodulin-Ca^{++}-R24571 state at low Ca^{++}. R24571 is apparently unique among the drugs tested in that at low concentrations it can increase the affinity of CaM for calcium so that Ca^{++} dependent felodipine binding can occur even at pCa 7.0.

In the following model (see Scheme 1) we account for the Ca^{++} dependent allosteric interactions among prenylamine, R24571, and felodipine. Kuo (106) has shown that, in the Monod-Wyman Changeaux (107) model, cooperative ligands could be both activators and inhibitors. The sigmoidal curve (Hill coeff. = 2.0) of a cooperative ligand shifts to a hyperbolic curve (Hill coeff. = 1.0) with enhanced binding at low ligand concentration in the presence of a second cooperative ligand. This is analogous to what we observe with R24571 and prenylamine, respectively, in their enhancement of felodipine binding. Noncooperative ligands cannot, by this model, produce enhancement. Therefore, enhancement of felodipine binding implies
Prenylamine cannot be explained by a simple two state Ca$_4$$^{++}$-calmodulin model. This apparent negative cooperativity can best be explained by prenylamine's ability to bind to an active Ca$_3$$^{++}$-calmodulin conformer, which is in equilibrium with an active Ca$_4$$^{++}$-calmodulin conformer (Scheme 1). This would keep the concentration of the Ca$_4$$^{++}$-calmodulin conformer relatively constant and produce apparent negative cooperativity (Hill coefficient=0.6) when calmodulin was titrated with felodipine in the presence of prenylamine. This two active conformer model could also explain the broad curve observed when calmodulin-felodipine is titrated with prenylamine (Hill coefficient=0.8) by the same mechanism.

A free energy coupling model in which both Ca$_3$$^{++}$-calmodulin and Ca$_4$$^{++}$-calmodulin have active conformations is the simplest model to explain the observed results (see Scheme 1). In this model prenylamine could bind the Ca$_3$$^{++}$-calmodulin conformer almost as well as to Ca$_4$$^{++}$-calmodulin conformer whereas diltiazem, R24571 and felodipine would have a greater preference for the Ca$_4$$^{++}$-calmodulin conformer. When calmodulin and felodipine are titrated with Ca$^{++}$ a Hill coefficient of 4 is observed, consistent with Ca$_4$$^{++}$-calmodulin being the active species. Ca$^{++}$ titrations of CaM in the presence of both felodipine and prenylamine exhibit Hill coefficients of 3 or less, consistent with prenylamine binding to Ca$_3$$^{++}$-calmodulin (i.e. for prenylamine $K_r$ $K_t$; for R24571, diltiazem and felodipine $K_r$<<$K_t$).

The equation for drug binding when $K_r$<<$K_t$ and S/$K_r$>>1 is:
This is analogous to the Hill equation and $K_{0.5}$ from the Hill plot:

$$(L_a)^{1/2} \cdot Kr.$$  

The maximum value for $Kr$ is similar to the 0.5 μM value determined from a titration of felodipine with calmodulin (CaM) in the presence of R24571 or prenylamine (Fig. 3). The minimum value for $La$ then is 1700 ($\Delta G = +5$ Kcal/mole). The Hill plot is a reasonable approximation for $S/Kr > 10$ or for $S > 5$ μM.

At low Ca++ concentration the Ca$_3^{++}$-CaM-R24571 species may predominate over Ca$_4^{++}$-CaM-R24571 producing apparent noncompetitive inhibition at low calcium concentrations, ($B_{max}$ is reduced from 1.8 to 0.8 mol/mol); an analogous situation is seen with prenylamine at pCa=3 (Fig. 2B). This could account for the biphasic Ca++ titration curve seen in the presence of R24571 (Fig. 7). The first phase of the fluorescence increase probably represents Ca++ induced formation of the conformer Ca$_3^{++}$-CaM with two drugs (D$_2$). The final increase probably represents the addition of calcium to this species to form Ca$_4^{++}$-CaM with two drugs. The biphasic increases in fluorescence intensity observed in this Ca++ titration would represent sequential increases in drug affinity or free energy of the system as shown in scheme 1.

This model is consistent with the observation of Keller et al. (108) who observed that TFP enhanced the binding of the first three Ca++, but inhibited binding of the fourth, possibly indicative of preferential binding to Ca$_3^{++}$-CaM. Analinonaphthalenesulfonate, on the other hand, increased the cooperativity of Ca++ binding to all four Ca++ sites.
The model we propose for Ca\(^{++}\) dependent binding of drugs is similar to those proposed for calmodulin binding to its target proteins. In addition, our model accounts for the cooperative binding among drug/protein binding sites exposed on different Ca\(^{++}\) conformers. In a review of the calcium dependence of calmodulin's activation of various proteins, Cox (109) has shown that Ca\(^{3+}\)-CaM and/or Ca\(^{4+}\)-CaM are the active species. Ca\(^{4+}\)-CaM is apparently the active species for activating the calmodulin dependent protein kinase of cardiac sarcoplasmic reticulum (49) and its activation by Ca\(^{++}\) displays a Hill coefficient of 4. For phosphorylase kinase, however, Ca\(^{3+}\)-CaM is the active species (45), with both the tightly bound and loosely bound calmodulin molecules binding three Ca\(^{++}\) in the active state, with each exhibiting distinct Ca\(^{++}\) titration curves. This is consistent with my model's two conformational states for Ca\(^{3+}\)-CaM, each of which should exhibit distinct Ca\(^{++}\) titration curves. These studies of CaM-protein interaction are analogous to my finding that Ca\(^{3+}\)-CaM and Ca\(^{4+}\)-CaM are the active species for drug binding. If this is true, then calmodulin antagonist drugs might be expected to mimic the effects we observe on felodipine binding. Metzger et al. (110) have shown that prenylamine is able to enhance and then inhibit the velocity of contraction of skinned smooth muscle. Maximal potentiation is observed at 10 \(\mu M\) prenylamine, while higher prenylamine concentrations produce inhibition. The contraction of chemically skinned smooth muscle is dependent on the calcium dependent interaction and activation of myosin light chain kinase (MLCK). This suggests that prenylamine is able to regulate the binding of
myosin light chain kinase (MLCK) to calmodulin in a manner analogous to its allosteric regulation of felodipine binding.

Further, my studies indicate that one calmodulin antagonist (R24571) can dramatically enhance the apparent affinity of CaM for Ca++. Similarly, it has been shown that troponin I (55), MLCK, (35), and melittin (111) binding to calmodulin enhances its Ca++ affinity. In this respect also, drug binding seems analogous to protein binding. My allosteric model suggests that various drugs may inhibit (or stimulate) calmodulin-protein interactions differentially. Consistent with this, Hidaka et al. (89) have shown that prenylamine inhibits in the order PDE > Ca++Mg++ATPase > MLCK whereas W-7 inhibits in the order Ca++Mg++ATPase > MLCK > PDE, indicating that various drugs can show some selectivity in inhibiting various targets. Moreover, his data also showed very little correlation between inhibition of $^3$H W-7 binding and enzyme inhibition, indicating that simple competition cannot account for the observed inhibition of enzymes. This and my data clearly suggest that stricter analysis of drug inhibition data is necessary to determine if some of the CaM-ANTS drugs may allosterically or competitively affect various CaM-protein interactions.

My finding of two binding sites for felodipine is in agreement with Jarret et al. (61) who labeled calmodulin with the affinity reagent 10-(3 aminopropyl)-2-(trifluoromethyl) phenothiazine and found that 2 moles of reagent were covalently coupled per mole of calmodulin. Newton et al. (112) showed that only one mole of 2-chloro-10-(3 isothiocyanatopropyl) phenothiazine was incorporated
into calmodulin but that the modified calmodulin was able to bind to cyclic nucleotide phosphodiesterase and inhibit its activation by unmodified calmodulin. They also observed increased rates of incorporation of affinity label when TFP was added at low concentrations, consistent with our observation of allosteric interaction among calmodulin's drug binding sites. Affinity labels should be useful in determining the effects of drug binding on Ca++ affinity without the interference of multiple equilibria, as well as for determination of K_r, the dissociation constant of the activated state for various drugs.

Finally, the model proposed here has much in common with what is known for hemoglobins (113). The effect of prenylamine and R24571 on felodipine binding is similar to known effects of CO on hemoglobin where CO enhances hemoglobin affinity for O_2 at low oxygen concentrations and displaces it at high oxygen concentrations (114). If drug (or protein) binding can liberate a proton from calmodulin similarly to that which occurs when O_2 binds to hemoglobin, this might explain how Ca++ affinity is increased when drugs or proteins bind to calmodulin. Also, there might be naturally occurring allosteric regulators of calmodulin, analogous to those known for hemoglobin, which may alter its sensitivity to Ca++ and/or impart selectivity for calmodulin binding to its target proteins.
Fig. 1. Correlation of the fluorescence increase observed in felodipine with its binding to CaM. The change in felodipine fluorescence is plotted vs. 3H felodipine binding in the presence (○-○) and absence (▲-▲) of 10 μM prenylamine. Equilibrium dialysis was conducted in Buffer A with 1 μM calmodulin and variable (0.5 - 5 μM) felodipine.
Fig. 2. (A) Scatchard analysis (B/S2-vs-B) from fluorescence data of felodipine titrations of 1 μM calmodulin. Bound felodipine was determined from a standard curve of felodipine fluorescence-vs-3H-felodipine bound as in Fig. 1. Buffer was 4% v/v ethanol in Buffer A. (B) Scatchard analysis (B/S vs B) in the presence of 10 μM prenylamine (□-□) or 2 μM R24571 (○-○). Excitation was at 365 nm and felodipine emission was measured at 445 nM.
Fig. 3. Hill plot analysis of fluorescence enhancement observed when 1 μM calmodulin is titrated with felodipine alone (▲-▲), $K_{0.5} = 22$ μM, slope = 1.9, $r = 0.997$; in the presence of 2 μM R24571 (○-○), $K_{0.5} = 15$ μM, slope = 1.0, $r = 0.993$; or in the presence of 10 μM prenylamine (■-■), $K_{0.5} = 58$ μM, slope = 0.6, $r = 0.993$. For felodipine alone, only those points where $S > 5$μM were used in determination of $B_{max}$ and the Hill coefficient.
Fig. 4. Hill plot analysis of fluorescence enhancement of felodipine titrated with calmodulin alone (○-○) or in the presence of 2 μM R24571 (▲-▲) or 10 μM prenylamine (□-□). The cuvette contained 0.5 μM felodipine in buffer A and [CaM] represents total calmodulin added.
FIGURE 5: (Upper panel) The fluorescence changes produced by titrations of calmodulin–felodipine with R24571 (■), prenylamine (○), and diltiazem (●) are compared with a theoretical curve. The solid line (T) is the curve expected if felodipine binds to calmodulin to which one and only one molecule of potentiating drug has bound to one of two equivalent sites on calmodulin ($K_d = 10.7 \mu M$). The F curve (▲), a plot of percent bound vs. bound at 1 mM felodipine, represents a titration of 1 mM felodipine and 1 mM calmodulin with a drug that is identical to felodipine but is nonfluorescent. The drug titrations were conducted with 0.2 μM calmodulin and 1 mM felodipine in buffer A. (Lower panel) Plots of the integrals of the theoretical (—), felodipine (▲), R24571 (■), prenylamine (○), and diltiazem (●) curves shown in the upper panel. Hill plots of the integral of the theoretical curve gave a slope of 1.0, $r = 0.9999$, with $K_{0.5} = 9.7 \mu M$. Hill coefficients were 1.8 ($r = 0.990$) for R24571, 2.0 ($r = 0.999$) for diltiazem, and 0.8 ($r = 0.999$) for prenylamine titrations of calmodulin–felodipine. The slope and $K_{0.5}$ determined for the curve of felodipine alone were nearly identical with those experimentally obtained in Figure 3 ($K_d = 22 \mu M$, slope = 2.0), verifying integration by the trapezoidal method.
Fig. 6. Scatchard analysis of felodipine binding to calmodulin in the presence of R24571 at various calcium concentrations. 1 μM calmodulin and 2 μM R24571 were titrated with felodipine in 10 mM MOPS, 90 mM KCl, 2 mM EGTA, 4% ethanol with free calcium adjusted to pCa 7.0 (Δ-Δ), pCa 4.0 (●-●) or pCa 3.0 (○-○). K0.5 were 10 μM (r = 0.94), 12 μM (r = 0.98) and 12 μM (r = 0.97), respectively. The inset shows the fluorescence increase produced when 0.5 μM calmodulin and 1 μm felodipine are titrated with R24571. A Hill plot of the integral showed K0.5=1μM: Hill coefficient=2.0; r=.998
Fig 7. Calcium titrations of 2 μM calmodulin and 1 μM felodipine (Δ-Δ); calmodulin, felodipine and 10 μM prenylamine (○-○); or calmodulin, felodipine, and 2 μM R24571 (■-■). Hill coefficient with felodipine alone was 3.8 ± 0.3; r = 0.993 (average of 5 determinations). Hill coefficient for felodipine and prenylamine = 2.5 ± 0.1; r = 0.998 (average of 3 determinations). Buffer contained 10 mM MOPS 90 mM KCl, and 2 mM EGTA, pH 7.0, and CaCl₂ was added to give the free calcium indicated.
Fig. 8. Model for calmodulin interactions with calcium and drugs. La, and Lb represent the ratio of inactive species to active species for Ca4CaM and Ca3CaM respectively. Kr and Kt are dissociation constants. D, drug; (●) binding site occupied with calcium; (■) and (>), unoccupied exposed drug binding sites of distinct conformations; (■) and (●), occupied binding sites of these distinct conformations.
2. METAL IONS AS ALLOSTERIC REGULATORS OF CALMODULIN

Introduction
Calmodulin is an ubiquitous calcium binding protein that has now been implicated in conferring calcium sensitivity to over thirty different target proteins (see 4,7-9, for review). With calcium binding to the four calcium binding sites on calmodulin, large structural changes including increases in α-helix and intrinsic tyrosine fluorescence occur. Perhaps the most significant calcium dependent event to occur on the surface of calmodulin is the exposure or formation of hydrophobic drug/protein binding sites (125-126). These interfacial sites are thought to be the sites where calmodulin interacts with its target proteins and with inhibitory drugs (calmodulin antagonists). Felodipine, [4-(2,3-dichlorophenyl)-1,4 dihydropyridine-2,6-dimethyl 3-5-dicarboxylic 3-ethylester and 5-methylester], is perhaps the most potent member of the powerful dihydropyridine calcium channel blocking drugs (127-128). Unlike most other therapeutically useful dihydropyridines, felodipine is fluorescent. It binds to calcium channels on purified sarcolemma fractions with affinities in the subnanomolar range and has recently been shown to bind to calmodulin in a calcium dependent manner (24,128). It has been suggested that felodipine's interaction with calmodulin may contribute to its potency as a vasodilator (129). In chapter 1, I showed that some of these calcium dependent drug binding sites are allosterically related and that drug binding to some site(s) can potentiate felodipine binding to a distinct site by producing a twenty fold increase in its apparent affinity. I also
showed that the fluorescence increase which occurs with the binding of felodipine provides an accurate means of monitoring these allosteric interactions among calmodulin's drug binding sites.

In addition to drug binding sites, calmodulin has metal binding sites. Not only calcium but La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$, Sm$^{3+}$, Sr$^{2+}$, Hg$^{2+}$, Cd$^{2+}$, Zn$^{2+}$ and Mn$^{2+}$ have been shown to interact with calmodulin (115-118). Some of these metals can compete for the calcium binding sites on calmodulin while others may have effects on calmodulin's conformation beyond those produced by calcium alone (118). Recently, calmodulin has been suggested to be a mediator of some of the toxic effects of these metals in heavy metal toxicity (117). Several authors have shown that some of these metals can activate calmodulin dependent phosphodiesterase in the absence of calcium (117,121) while others may inhibit in the presence of calcium (71,72). Moreover, a close correlation between metal toxicity and metal binding to calmodulin has been demonstrated (72). In addition, many of these metals affect lymphocyte mitogenesis (122), a process known to be calcium dependent (123).

In this chapter I will examine the effects of metal cations, including La$^{3+}$, Tb$^{3+}$, Cd$^{2+}$, Pb$^{2+}$, Zn$^{2+}$ and H$^{+}$ in producing the conformation of calmodulin which is allosterically potentiated for binding felodipine. Further, I examine the effects of Hg$^{2+}$ and Cu$^{2+}$ in displacing these other metals and inactivating this active conformer of calmodulin.

My findings suggest that if interactions of some of these metals and calmodulin occur in vivo, they could dramatically affect calmodulin's regulation of calcium dependent events within the cell.
and produce significant biological consequences.

**Materials and methods**

**Equilibrium dialysis** - Measurements were conducted in a buffer of 10 mM Mops pH 7.0, 90 mM KCl with 2 mM EGTA and 3 mM Ca\(^{2+}\) or 1 mM La\(^{3+}\) and 1 mM Ca\(^{2+}\). \(^{3}H\)-felodipine of constant specific activity was added to the solution (50 ml) outside the dialysis bag which contained 3 ml of 1 \(\mu\)M calmodulin. Specific activity was determined using a millimolar extinction coefficient at 370 nm of 6.4 in 95% ethanol.

**Fluorescence studies** - Buffers were sufficiently free of Ca\(^{2+}\) so as not to produce any fluorescence increase in the presence of both calmodulin and felodipine. Since we have previously shown that half maximal felodipine binding occurs at 1 \(\mu\)M Ca\(^{2+}\), free Ca\(^{2+}\) was less than 1 \(\mu\)M. This was verified by atomic absorption spectroscopy. Fluorescence titrations were carried out in 10 mM MOPS, 90 mM KCl pH 7.0 unless otherwise indicated using excitation at 365 nM and emission at 445 nM.

Hill plots were analyzed by an iterative linear regression Hill plot program. \(B_{\text{max}}\) was determined by the best fit of the data to the Hill equation. Felodipine and \(^{3}H\)-felodipine were gifts of A.B. Hässle Pharmaceutical, Mölndal, Sweden. All other chemicals were reagent grade. Calmodulin was purified from bovine testes as previously described (105).

**Results**

Previously, we have shown two felodipine binding sites on calmodulin. These sites exhibit cooperativity in their drug binding. Calmodulin antagonists (including prenylamine and R24571) bind to
calmodulin and allosterically potentiate felodipine binding to its remaining site by producing a large increase in its affinity. Some metals are capable of producing similar enhancements of felodipine binding and felodipine-calmodulin fluorescence. The inset of Fig. 9 shows the fluorescence spectra of felodipine in the presence of calmodulin (spectra 1), after the addition of 1 mM calcium (spectra 2), and after the subsequent addition of 300 μM lanthanum chloride. Calcium produces a 40% increase in felodipine-calmodulin fluorescence, and the subsequent addition of lanthanum produces a large additional 4.0 fold increase in fluorescence. In the absence of added calcium, lanthanum alone produces a 6.2 fold increase in fluorescence with the concentration dependence shown in Figure 9. Lead and cadmium produce similar enhancements, both in the presence and in the absence of added calcium, with the concentration dependence shown in Figure 9. Zinc produces a large enhancement in felodipine-calmodulin fluorescence only in the presence of added calcium. The concentration dependence of this zinc-induced increase in fluorescence in the presence of 1mM calcium is shown in Figure 9. Similar to lanthanum, terbium produces a 7.0 fold fluorescence increase in the absence of calcium which is half-maximal at 12 μM and a 6.2 fold increase in the presence of 1 mM calcium which is half-maximal at 12 μM (data not shown). Thus, lanthanum, terbium, lead and cadmium (in their order of effectiveness) potentiate felodipine binding (fluorescence) both in the presence or absence of added calcium, while zinc is stimulatory only in the presence of calcium. Manganese is similar to zinc, producing a smaller fluorescence increase (1.6 fold), which is half-maximal at 1mM
and occurs only in the presence of calcium (data not shown). BaCl$_2$, MgCl$_2$, NaCl, KCl, and RbCl are all ineffective (at concentrations up to 1mM) in affecting felodipine-calmodulin fluorescence either in the presence or absence of calcium. None of the metals produce any change in felodipine fluorescence in the absence of calmodulin.

In chapter 1, I showed that the fluorescence increase produced by felodipine binding to calmodulin can be used to quantitate felodipine binding in the presence and absence of potentiating drugs. A similar direct correlation between $^3$H-felodipine binding and the felodipine fluorescence increase produced by binding is shown in Fig. 10 (in the presence and absence of lanthanum). An eight fold increase in fluorescence corresponds to one mol felodipine binding per mol of calmodulin. Thus the increase in felodipine fluorescence produced by these potentiating metals is due simply to an increase in calmodulin's affinity for felodipine and therefore more felodipine molecules binding to calmodulin; as verified by these $^3$H-felodipine binding studies. This figure was used as a standard curve to quantitate felodipine binding in the following fluorescence experiments.

Hill plots of felodipine titrations of calmodulin alone, and calmodulin potentiated by zinc+calcium or by lanthanum+calcium are shown in Figure 11. Felodipine alone exhibits a Hill coefficient of 2.1 ($r=0.999$), indicative of cooperativity among the two felodipine binding sites. A Bmax of 2.0 mol/mol was determined by best fit analysis of this data and a K of 8 μM was found. In the presence of zinc+calcium or lanthanum+calcium, the Hill coefficient was reduced to 0.96 ($r=0.98$) or 0.83 ($r=0.97$ with K 0.5 of 0.5 μM and 0.2 μM),
respectively. A Bmax of 2.0 was determined with both zinc and lanthanum. These potentiating metals abolish the cooperativity among calmodulin's two felodipine binding sites. Calmodulin titrations of felodipine with and without a metal were used to determine the effect of potentiating metals on calmodulin's affinity for felodipine. In the absence of metal, calmodulin binds felodipine with a K0.5 of 8.0 μM while in the presence of zinc+calcium or lanthanum the K0.5 is decreased approximately 20 fold to 0.4 μM (Fig. 12). These potentiating metals, therefore, produce a very dramatic increase in calmodulin's affinity for felodipine.

Zinc is the only metal which requires calcium before it will dramatically potentiate felodipine binding to calmodulin. This allowed the determination of the calcium dependence of zinc potentiation. Calcium titrations of felodipine-calmodulin at various concentrations of zinc are shown in Fig. 13. The calcium dependence is approximately the same at concentrations of zinc which produce 1/4 maximal, 1/2 maximal and maximal stimulation of felodipine binding. The similarity of the calcium dependence of potentiation over a wide range of zinc concentrations suggests that zinc is not very competitive with calcium at the calcium binding sites. Zinc titrations of felodipine-calmodulin at 1mM and 50 μM calcium and at 5mM strontium are shown in Fig. 14. At lower calcium concentrations (50 μM) zinc potentiates felodipine fluorescence at low concentrations but inhibits it at higher zinc. At higher calcium (1mM), zinc only potentiates felodipine fluorescence over the range tested. This suggests that zinc, at a higher concentration, is able to compete with calcium (at lower concentrations), displacing it from calmodulin and
preventing felodipine binding and fluorescence enhancement. At higher calcium concentrations (1mM), zinc is less able to effectively displace calcium and produce this fluorescence decrease. Strontium is known to substitute for calcium on calmodulin (40). It has a lower affinity for calmodulin and produces a small increase in felodipine-calmodulin fluorescence (1.2 fold) similar to calcium. Zinc initially potentiates this strontium-supported increase in fluorescence and then displaces strontium to prevent felodipine binding at higher zinc concentrations (see Fig 14). This data suggests that high concentrations of zinc can compete with the calcium (and strontium) binding sites on calmodulin, under conditions of low calcium. Thus, while calcium cannot effectively compete with the zinc binding site(s) which produce potentiation, high concentrations of zinc can compete with the calcium sites on calmodulin.

Copper and mercury produce no potentiation of felodipine-calmodulin fluorescence either in the absence or presence of calcium. Instead, they decrease the fluorescence increases produced by the potentiating metals. Figure 15 shows the effects of copper and mercury in reversing the potentiated states produced by zinc+calcium, lanthanum and lanthanum+calcium. Copper half-maximally inhibits each of these potentiated states near 50 μM. Mercury is less effective, half-maximally inhibiting each of these potentiated states near 2 mM. The concentration range over which both copper and mercury produce the reversal of the lanthanum potentiated state is essentially the same in the presence of 1mM calcium as in the absence of added calcium. Similar results were found with lead. Copper half-maximally inhibits the lead-potentiated state (300 μM lead) at 50 μM both in the
presence and absence of 1 mM calcium. The calcium dependence of the zinc potentiated state (3.6 mM zinc) is not affected (half-maximal 50 μM calcium) by the presence of 50 μM copper, while this concentration of copper does reduce the total amount of felodipine bound in the presence of zinc (F/Fo=2.3). Further, in the presence of sub-maximal potentiating metal (35 μM Pb²⁺ and 15 μM La³⁺), copper is more effective in inactivating the potentiated state (half maximal at 14 μM copper) than in the presence of higher concentrations of potentiator (see Fig. 14). Similar results were found either in the presence or absence of 1 mM added calcium. Moreover, 50 μM copper and 2 mM mercury shift the concentration dependence of the lanthanum induced increase to the right from 8 μM (as in Fig. 9) to 50 μM and 60 μM, respectively. This data is consistent with both copper and mercury binding competitively at the potentiating metal binding site(s) and not at the calcium binding sites of calmodulin.

Calcium itself at concentrations well above where it binds to the calcium binding sites on calmodulin is able to produce a potentiation of felodipine binding. Calcium at concentrations above the millimolar range produces a 5 fold increase in calmodulin-felodipine fluorescence which is half-maximal at 50 mM calcium. This suggests that calcium is 5000 fold less effective than La³⁺ and 100 fold less effective than zinc in binding to those potentiating sites on calmodulin.

Protons are also able to produce this highly fluorescent potentiated state of felodipine-calmodulin. Figure 16 shows pH titrations of felodipine and of felodipine-calmodulin in the presence and absence of calcium. Felodipine alone and felodipine-calmodulin in
the absence of calcium were not affected by pH over the range tested. Titrations of felodipine-calmodulin in the presence of calcium, however, showed a sharp pH dependence, with maximal potentiation near pH 4.75. This data suggests that concentrations of protons in this range can act in a similar manner as some of the metal cations in potentiating felodipine binding to calmodulin. Felodipine titrations of calmodulin + calcium at pH 4.75 verified that this was the case. Hill plots of this data (not shown) indicated that the Hill coefficient was reduced from 2.0 at pH 7.0 to 0.96 (r=.996) at pH 4.75 with a Bmax of approximately 1.7 mol/mol and K0.5 = 4μM. Calmodulin titrations of felodipine at pH 4.75 indicate that the apparent affinity is increased approximately 14 fold relative to pH 7.0 (data not shown). Thus, protons are capable of abolishing the cooperativity among felodipine binding sites and increasing the affinity of calmodulin for felodipine in a manner analogous to metals and potentiating drugs. Calcium is more effective in producing an increase in calmodulin-felodipine fluorescence as the proton concentration is reduced. At pH 5.5, calcium produces a 2.7 fold increase (over felodipine alone) which is half-maximal at 3.5 μM while at pH 4.0, calcium produces a 2.9 fold increase which is half-maximal at 425 μM.
Discussion

In chapter 1, I showed that allosteric interactions occur among calmodulin's drug binding sites. Drugs, including the calmodulin inhibitors R24571 (calmidazolium), and prenylamine, can bind to one of the two felodipine binding sites on calmodulin, abolish the cooperativity among these sites, and increase the affinity of felodipine at its remaining binding site. The fluorescence of the dihydropyridine calcium channel blocker, felodipine, provides an accurate means of monitoring the binding of ligands which potentiate felodipine binding by allosterically increasing the number of calmodulin molecules in the active conformation.

In the present study, we describe the effects of various metals in potentiating felodipine binding to calmodulin. La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$ and Cd$^{2+}$ act in a manner analogous to calmodulin antagonists and dramatically potentiate felodipine binding to calmodulin. These metals cause potentiation over a similar concentration range in the absence of added calcium and in the presence of 1 mM calcium, suggesting that they stabilize this active conformer of calmodulin by binding to sites other than the calcium binding sites. Zinc also produces a strong potentiation but only in the presence of calcium. With each of these metals and with zinc + calcium, the fluorescence increase results from a large increase in the affinity of felodipine for calmodulin which occurs concomitant with the abolition of cooperativity among the two felodipine binding sites (Hill coefficient was reduced from 2.0 to 1.0). Titrations of felodipine with calmodulin indicate that the affinity of calmodulin for felodipine is
increased approximately twenty fold in the presence of potentiating metals.

In the case of calmodulin antagonist drug induced potentiation of felodipine binding, the potentiating drugs bind to calmodulin only in the presence of calcium and produce their potentiated state by binding to sites which are distinct from the calcium binding sites on calmodulin (77). The active conformer produced by these metals is kinetically identical to that produced by drugs + calcium. It is, therefore, likely that these metals require occupancy of the calcium binding sites before they can bind to distinct site(s) and produce this active conformation of calmodulin. With zinc, the requirement for calcium on the calcium binding sites before zinc can bind to the potentiation site is obvious. La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$ and Cd$^{2+}$ can bind both to the calcium binding sites and the potentiating site(s) to produce potentiation in the absence of calcium. Consistent with this Chao et al. (21) have shown that La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$, and Cd$^{2+}$ are all effective in displacing calcium from calmodulin, promoting an increase in its intrinsic tyrosine fluorescence and modulating its activation of phosphodiesterase. Zinc and manganese, on the other hand, are not very effective in displacing calcium and producing the structural changes produced by calcium binding (21). Zinc and manganese, therefore, require calcium before they can produce the active calmodulin conformer. Occupancy of the calcium binding sites by La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$, Cd$^{2+}$ or by calcium appears to be necessary before any of these metals can bind to the sites where they produce potentiation.
Our findings suggest that potentiating metals including La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$ and Cd$^{2+}$ bind to sites which are distinct from the calcium binding sites to produce an allosterically potentiated state of calmodulin. Copper and mercury compete with these metals at this site(s) and produce an inactivation of this active calmodulin conformer. Our evidence for these conclusions is as follows:

1. The concentration dependence of potentiation by La$^{3+}$, Tb$^{3+}$, Pb$^{2+}$, and Cd$^{2+}$ is not shifted by the presence or absence of 1 mM calcium.

2. Potentiation by zinc requires calcium, suggesting that distinct sites are involved for calcium binding and for potentiation.

3. The calcium dependence of potentiation by zinc is similar over a wide range of zinc concentrations, suggesting that over the range where zinc potentiates it does not compete for the calcium binding sites.

4. Chao et al. (21) have shown that Pb$^{2+}$ and Cd$^{2+}$ are equally effective in displacing calcium from calmodulin, yet Pb$^{2+}$ is more than 50-fold more effective than Cd$^{2+}$ in potentiating felodipine binding.

5. Copper and mercury inactivate the La$^{3+}$, Pb$^{2+}$ and Zn$^{2+}$ potentiated state over the same concentration range irrespective of calcium concentration, suggesting that these metals compete for the metal ion potentiating site and not the calcium binding sites.

6. The concentration dependence of La$^{3+}$ potentiation is shifted to the right by the presence of Cu$^{2+}$ and Hg$^{2+}$, suggesting that Cu$^{2+}$ and Hg$^{2+}$ compete with the La$^{3+}$ potentiating site and not the calcium binding sites.
7. The calcium dependence of zinc potentiation is not shifted by the presence of half-maximally inhibiting copper (although the extent of potentiation is reduced), suggesting that copper inactivates by competing with the potentiating metal binding site (zinc) and not at the calcium binding site.

8. As the concentrations of potentiating metal (La$^{3+}$, Pb$^{2+}$) are reduced, Cu$^{2+}$ and Hg$^{2+}$ are more effective in inactivating the potentiated state, independent of the presence of 1 mM added calcium. Again, this suggests that Cu$^{2+}$ and Hg$^{2+}$ compete at the metal ion potentiating site and not at the calcium binding sites.

9. At very high concentration, calcium itself can produce potentiation of felodipine binding which is similar to that seen with much lower concentrations of these metal cations, suggesting that at supraphysiological concentrations, calcium will occupy these metal ion potentiating sites.

10. Studies of terbium binding to calmodulin indicate that over the same concentration range where lanthanum, lead, cadmium and zinc potentiate felodipine-calmodulin fluorescence (and over the range where copper decreases fluorescence), these metals will displace terbium (See Figure 17.) This is consistent with these potentiating metals binding at the same ion binding site on calmodulin.

The effect of protons on this active conformer of calmodulin are similar to the action of zinc. In the presence of calcium, protons produce an activation followed by an inactivation of the active conformer at lower pH. The curve shown in Figure 16 can be fit reasonably well assuming a maximum F/Fo of 8.0 (when all the molecules are in the active conformation), a pKa for activation of 5.15, and a
pKa for inactivation of 4.65. Using these parameters, the maximum number of molecules in the active state occurs at pH 4.9 where approximately 40% of the calmodulin molecules are in the active conformation. Activation presumably occurs with protonation of carboxyl groups on the protein with pKa 5.15. Inactivation presumably occurs by displacement of calcium by protons at lower pH. Consistent with this Andersson et al. (119) and Haiech et al. (20), have shown that protons can compete with calcium on calmodulin (pKa=4.4) in this range. Further, calcium is 120 fold more effective in producing an increase in felodipine-calmodulin fluorescence at pH 5.5 than at pH 4.0. These findings are consistent with proton competition with calcium on calmodulin at low pH and can explain the inactivation of the active conformer, by displacement of calcium, below pH 4.9.

This data indicates that some metals are capable of not only occupying the calcium binding sites of calmodulin, but also of supporting an allosterically potentiated conformer of calmodulin. This potentiation is produced by these metals binding to sites on calmodulin which are exposed or formed after calcium has bound. This active conformer is similar to the conformer produced by calcium and low concentrations of calmodulin antagonists (including R24571 and prenylamine), in that it has an enhanced affinity for felodipine and exhibits no cooperativity among its felodipine binding sites.

Activation of calmodulin in the cell is the consequence of increased intracellular calcium levels and perhaps the presence of putative endogenous regulatory substances. Together these may produce active calmodulin conformers for the selective activation of
calmodulin's various target proteins. If these metals were to obtain high enough concentrations intracellularly, then they might activate (or inactivate) calmodulin in a manner which was not controlled by calcium influx. Intracellular calcium dependent events would no longer be under their normal control mechanisms and many vital cellular processes could be dramatically altered. This notion is consistent with the proposal of Chao et al. (21) that heavy metal interaction with calmodulin may play a role in some types of metal toxicity. Further, Cox and Harrison (72) have demonstrated a high positive correlation between metal binding to calmodulin and their toxicity (LD50) in rats. Some of these metals (in particular Cd2+, Hg2+, and Pb2+, which are serious environmental pollutants) may accumulate to high concentrations in man and result in muscular, renal, bronchial and neurological disorders (124-129). They may act via binding to calmodulin and uncoupling it from its normal cellular control by calcium.

Finally, it should be mentioned that other calcium binding proteins including α-lactalbumin and β100 protein have been reported to have zinc binding sites which are distinct from their calcium binding sites. Similar to our findings with calmodulin, metal occupancy of these sites produces conformations of these proteins which were distinct from those produced by calcium binding to the calcium binding sites alone (130-131). The role of these metal ion binding sites in the in vivo regulation of the action of calcium binding proteins is as yet uncertain.
Figure 9. The effect of various metals on calmodulin-felodipine fluorescence. One μM felodipine and one μM calmodulin were titrated with each metal in the presence of 1mM CaCl2 or in the absence of calcium, in 10 mM MOPS, 90 mM KCl at pH 7.0. The percent of the total fluorescence changes is shown as a function of total metal added for LaCl3 with (○-○) and without (○-○) calcium; PbAc2 with (△-△) and without (△-△) calcium; ZnCl2 with (●-●) and without (●-●) calcium; and CdCl2 with (■-■) and without (■-■) calcium. In each case the addition of calcium produced a 40% fluorescence increase. Lanthanum produced a 6.2 fold fluorescence increase without calcium and a 4.0 fold increase above the fluorescence with calcium alone. Lead produced a 7.2 fold fluorescence increase and a 6.1 fold increase over calcium. Cadmium produced a 6.6 fold fluorescence increase and a 5.4 fold increase over calcium alone. Zinc produced a 4.5 fold fluorescence increase over calcium alone and no fluorescence increase in the absence of calcium. Excitation and emission wavelengths were at 365 nm and 445 nm, respectively.
Figure 10. The correlation between 3H felodipine binding and its fluorescence enhancement upon binding to felodipine. Felodipine titrations in the absence (O) and in the presence (▲) of 1.0 mM lanthanum are shown. This plot was used as a standard curve for determining the amount of felodipine bound in the subsequent fluorescence studies with an eight fold fluorescence increase occurring per mol of fel bound. Equilibrium dialysis was conducted as described in the Experimental procedures.
Figure 11. Hill plots of felodipine binding to calmodulin alone (▲) and in the presence of zinc (●), or in the presence of lanthanum (■). Each cuvette contained 0.5 μM calmodulin (except for the felodipine alone case where 1μM calmodulin was titrated) and either 3 mM zinc or 300 μM lanthanum in 10 mM MOPS, 90 mM KCl, 1mM CaCl2 at pH 7.0 with various concentrations of felodipine. Each point represents the average of three experiments.
Figure 12. Calmodulin titrations of felodipine alone (■-■), and in the presence of lanthanum (O-O) and in the presence of zinc (▲-▲). Buffer was the same as that used in Fig. 3 with 0.5 μM felodipine. The total calmodulin added is shown as the abscissa. Hill coefficients of calmodulin binding to felodipine of 0.9, r=.98; 1.2, r=0.99; and 1.0, r=0.99, were determined for felodipine alone, and in the presence of 300 μM lanthanum or 5mM zinc, respectively. The total fluorescence change was 2.4 fold for felodipine alone, 10.4 fold for +lanthanum and 7.8 for +zinc.
Figure 13. Effect of calcium on the zinc mediated potentiation of calmodulin-felodipine fluorescence. The fluorescence increase over the fluorescence of felodipine alone (F/Fo), in the presence of 230 µM zinc (▲▲), 575 µM zinc (○○), and 3.65 mM zinc (■■), is shown as a function of added calcium. The buffer was the same as in Fig 3. with 1 µM calmodulin and 1 µM felodipine.
Figure 14. The effect of zinc in potentiating calmodulin-felodipine fluorescence at different calcium and strontium concentrations. The total fluorescence change is shown as a function of added zinc for 1mM calcium (△-△), 50 μM calcium (Δ-Δ), and 5mM strontium (○-○). The buffer was that used in Fig 3. with 1 μM felodipine and 1 μM calmodulin.
Figure 15. The effect of copper and mercury in reversing the highly fluorescent potentiated states produced by lanthanum and zinc. Copper titrations of 1 μM calmodulin and 1 μM felodipine in the presence of 500 μM lanthanum alone (Δ-Δ), 500 μM lanthanum + 1 mM calcium (■-■) and 5 mM zinc + 1 mM calcium (▲-▲) and mercury titrations of 500 μM lanthanum alone (■-■), 500 μM lanthanum + 1 mM calcium (▼-▼) and 5 mM zinc + 1 mM calcium (〇-〇). In each case copper and mercury completely reversed the fluorescence increase produced by the potentiating metals. Buffer was as in Fig. 3.
Figure 16. Effect of pH on calmodulin-felodipine fluorescence. Each pH point was determined in a buffer of 20 mM Tris, 20 mM Mops adjusted to the appropriate pH by acetic acid or KOH. Felodipine (1μM) was then added (○-○) followed by 1μM calmodulin (△-△) and 1 mM calcium (■-■) and fluorescence measurements were taken at each pH.
Fig. 17. Analysis of terbium binding to calmodulin indicate that over the same concentration range where lanthanum, lead, cadmium, and zinc potentiate felodipine-calmodulin fluorescence (and over the same range where copper decreases fluorescence), these metals will displace terbium from calmodulin.
3. PREPARATION OF BIOLOGICALLY ACTIVE FLUORESCENT DERIVATIVES OF SPINACH CALMODULIN

Introduction

Calmodulin is a highly conserved protein which interacts with a large number of target proteins in a calcium dependent manner (for review see 4-6). Vertebrate calmodulin contains no cysteine, no tryptophan and two tyrosine (132). In order to study binding of calmodulin to target proteins in the nanomolar range, it is necessary to label it with highly fluorescent labels. However, since vertebrate calmodulin lacks cysteine it is difficult to label in a specific manner. 5-Iodoacetlyethylaminonapthalene-1-sulfonic acid has been used to label vertebrate calmodulin at methionines (133), but it was found necessary to affinity purify the fluorescently labeled calmodulin to obtain reliable results, since small amounts of unlabeled calmodulin markedly interfered with kinetic determinations. Diethylaminonapthlene-1-sulfonic acid calmodulin has been used to monitor interactions with calmodulin's target proteins, but was not sufficiently fluorescent to determine dissociation constants (134). Spinach calmodulin has been recently sequenced, and shown to contain a single cysteine residue at position 26 (135,136). Since it appears to activate some target proteins in a manner identical to vertebrate calmodulin, it represents an ideal candidate for specific fluorescent labeling with sulfhydryl selective fluorescent probes.

In this chapter, we describe the preparation and properties of maleimidylanilinonapthalene sulphonate (MIANS) spinach calmodulin and
Its interactions with three target proteins, smooth muscle myosin light chain kinase, smooth muscle caldesmon, and brain calcineurin. In addition, several other fluorescent spinach calmodulins have been prepared and their spectral characteristics determined. One of these, the highly fluorescent rhodamine X-maleimide derivative, has been used to determine the dissociation constant for calcineurin which was too low to be determined using MIANS calmodulin.

Materials and Methods

Materials

2-4' maleimidylanilinonapthalene-6-sulfonic acid (MIANS), 6-acryloyl-2-dimethylaminonaphthalene (acrylodan), 2,5 dimethoxystilbene-4'-maleimide (DMSM) rhodamine-X-maleimide, and 2' (N-methylanthraniolyl)-cGMP were obtained from Molecular Probes (Eugene, Oregon). All other chemicals were reagent grade. Fractogel DEAE was obtained from Rannin Instrument Corp. and phenyl sepharose C1 4B was obtained from Sigma Chemical Co. Calcineurin and cyclic nucleotide phosphodiesterase were purified by the method of Sharma et al. (137) through the calmodulin sepharose step. Myosin light chain kinase and caldesmon were purified as described previously (44,138).

Purification of Spinach Calmodulin and Fluorescent Labeling

Spinach calmodulin was purified using the method of Watterson et al. (135), with the following modifications. DEAE purification was done on Fractogel-DEAE using 10 mM MOPS pH 7, instead of tris pH 8.0 on DEAE-Sephadex. The eluted calmodulin was then purified on phenyl
sepharose CL 4B as described by Gopalakrishna and Anderson (97) in 10 mM MOPS pH 7.0. The sample was concentrated on a small Fractogel DEAE column and eluted with 0.5 M NaCl + 10 mM MOPS pH 7.0 (all buffers contained 200 μM phenylmethylsulfonylfluoride). This step removed the EGTA as well as concentrated the sample. This sample was incubated with a 4 fold molar excess of either MIANS, acrylodan, DMSM, or rhodamine-X-maleimide at room temperature for 2 hrs in the presence of 2 mM EGTA. To remove unreacted reagent, the labeled sample was then diluted 1/5 with 10 mM MOPS pH 7 and placed on a small Fratogel DEAE column. The column was washed briefly with 2 ml of 10 mM MOPS pH 7.0 and the labeled sample eluted with 0.5 M NaCl, 10 mM MOPS pH 7.0, and 200 μM PMSF.

The incorporation under these conditions were 1 mol/mol for MIANS (Assuming $E_{320} = 20,000$), 1.0 mol/mol for acrylodan ($E_{360} = 12,900$, ref 138), 0.6 mole/mole ($E_{364} = 25,000$) for DMSM and 0.8 mole/mole for rhodamine-X-maleimide ($E_{590} = 85,000$).

Assay of Cyclic Nucleotide Phosphodiesterase and Calcineurin

Calcineurin was assayed using the fluorescent assay of Anthony et al. (140) Phosphodiesterase was assayed using 2' (N methyl aniloyl) cGMP as a substrate, and its hydrolysis was followed by monitoring the fluorescence decrease observed, when excited at 280 nM with emission at 450 nM (141).

Fluorescence Titrations

Titrations of MIANS-CaM with MLCK, caldesmon and calcinurin were carried out at room temperature in buffer containing 10 mM MOPS, pH
7.0, 90 mM KCl and 1 mM CaCl₂. For Scatchard analysis, binding was corrected to free target protein concentration and the fluorescence change was corrected for reduction of free CaM. Excitation and emission wavelength pairs of 320, 440 nM, and 550, 600 nM were used for MIANS and rhodamine x derivatives respectively. Ca++ titrations were carried out in 200 mM MOPS (pH 7.0), 90 mM KCl and 2 mM EGTA to prevent pH changes. Free Ca++ was calculated as reported previously (104). Tb+++ titrations were carried out in the phosphorescence mode with excitation at 230 nM and emission of 543 nM. Delay time was 1 msec and gate time was 1 msec. Under these conditions 100 nM calmodulin could be titrated with very good signal to noise ratios, and this concentration was used for the analysis of Tb+++ binding to calmodulin. All measurements were carried out in a Perkin Elmer LS5 spectrofluorometer.

Results

To determine whether the reaction of MIANS was specific for cysteine 26 on spinach calmodulin, we compared the reactivity of MIANS toward spinach and bovine brain calmodulin. The reaction of MIANS with sulfhydryl groups produces a large increase in fluorescence which is useful as an indices of sulfhydryl reactivity (139). Figure 18 shows that spinach, but not bovine brain calmodulin reacts with MIANS. Subsequent dialysis of each protein indicated that bovine brain calmodulin was not labeled while spinach calmodulin was labeled with 1:1 stoichiometry. It is unlikely that there is any reaction of MIANS with residues other than cysteine 26, since brain calmodulin contains no cysteine but is otherwise highly homologous with spinach.
calmodulin (136). In the absence of Ca\(^{++}\) (2 mM EGTA) the reaction is 5-10 fold faster than in the presence of 1 mM Ca\(^{++}\) (data not shown). Thus, Ca\(^{++}\) binding to spinach calmodulin appears to reduce sulfhydryl reactivity, consistent with the location of cysteine 26 in a calcium binding loop (142).

MIANS calmodulin undergoes small but reproducible changes upon binding Ca\(^{++}\). Figure 19 shows the calcium dependence of MIANS-CaM fluorescence. A 5% increase is observed at pCa = 6.6 followed by a 15% decrease which is essentially complete at pCa = 6.0. Similar Ca\(^{++}\) dependent changes were seen with acrylodan-CaM with a 20% increase at pCa=6.8 and a 50% decrease by pCa=6.0 (data not shown).

Figure 20 shows titrations of MIANS-CaM with calcinurin, MLCK and caldesmon. Figure 4A shows a Scatchard plot of 5 nM MIANS spinach calmodulin with smooth muscle MLCK. MLCK binding produced a 4.6 fold fluorescence increase with a Kd of 9 nM. Free MLCK was determined assuming a 1:1 stoichiometry of CaM:Kinase. The inset shows the fluorescence spectra of 10 nM MIANS-CaM compared to buffer alone and also its spectra in the presence of a 4 fold molar excess of MLCK. Caldesmon binding to MIANS-CaM produces a 2-fold increase in fluorescence but its affinity is much lower (Kd = 250 nM). Calcineurin produced a 80% fluorescence increase with a Kd < 5 nM. MLCK, calcineurin and caldesmon produced no fluorescence change in the absence of Ca\(^{++}\) (data not shown). Melittin, a small peptide which binds to calmodulin with high affinity (143), produced no change in fluorescence at concentrations up to 3 \(\mu\)M (data not shown). In addition the binding of the calmodulin antagonist drugs calmidazolium (2 \(\mu\)M) and prenylamine (100 \(\mu\)M) did not affect MIANS fluorescence.
The most important criteria for a useful fluorescently labeled calmodulin is that it maintain its biological activity. Figure 22 shows the activation of cGMP phosphodiesterase (PDE) by both unlabeled and MIANS labeled spinach calmodulin. The labeling of CaM with MIANS does not result in any decrease in its ability to activate PDE. Acrylodan-CaM, DMSM-CaM, and rhodamine-X-CaM, like MIANS-CaM appears to be equieffective as unlabeled CaM in stimulating cGMP phosphodiesterase. MIANS and acrylodan labeled calmodulins were as effective as unlabeled calmodulin in activating calcineurin at one to one molar ratio (data not shown).

Several other sulfhydryl selective fluorescent probes were also used to label spinach calmodulin. Figure 23 shows the relative fluorescence of MIANS-CaM, DMSM-CaM, acrylodan-CaM, and rhodamine X-maleimide CaM. Although all derivatives are highly fluorescent, DMSM-CaM, and rhodamine-X-maleimide CaM are more fluorescent than MIANS-CaM and acrylodan-CaM. Since the binding of calcineurin to MIANS-CaM was essentially stoichiometric (Figure 20), the binding of calcineurin to subnanomolar concentrations of rhodamine X-CaM was investigated. Calcineurin titrations of 0.7 nM rhodamine X-CaM produced a 38% decrease in rhodamine-X-fluorescence. Scatchard analysis of this data indicated a Kd of 1.5 nM (r = .996). Rhodamine X-CaM gives a fluorescence twice that of background at 0.15 nM, so that it can be used to determine very high affinity binding. Because it exhibits high absorbption at the long wavelength lines of mercury and can be detected at subnanomolar levels, it should be ideal for use in the microscopic localization of calmodulin target proteins.
Discussion

Spinach calmodulin has been labeled with several sulfhydryl selective probes, presumably at cysteine 26. Consistent with the label being at cysteine 26 is our observation that the highly homologous brain calmodulin, which has a Thr for Cys at position 26 shows no reactivity with MIANS. Further, the maleimide reagent is quite specific for free SH groups at neutral pH (144). In addition, MIANS is incorporated into spinach-CaM with 1:1 stoichiometry. MIANS-CaM appears to retain biological activity since this modification produced no detectable change in its ability to activate either cGMP phosphodiesterase or calcineurin.

MIANS and Acrylodan labeled S-CaM undergo significant changes in fluorescence between pCa = 7 and pCa = 6.5. This change occurs at Ca++ concentrations where only small changes in tyrosine fluorescence are observed. Titrations of unlabeled and MIANS labeled S-CaM with terbium showed that labeling enhanced terbium affinity by 5-6 fold (data not shown). Thus the fluorescence changes at low calcium concentrations are probably due to an enhancement of calcium affinity by fluorescent labeling of cysteine 26. Recent stop flow studies have shown that site III and site IV are the high affinity Ca++ binding sites (145), while sites I and II exhibit lower affinity for calcium. Thus it is likely that Ca++ binding to sites III and site IV produce enhancement in MIANS (or acrylodan) fluorescence while binding at sites I and site II produce the subsequent decrease. However, it is possible that labeling may have altered the order of Ca++ binding. The recent availability of
genetically engineered calmodulins (101) which contain cysteine in both site I and site IV should allow this question to be addressed.

The interaction of MIANS-CaM with MLCK produces a 4.6 fold increase in its fluorescent yield with a Kd of 9 nM. This compares favorably with the reported Kd's of 1-4 nM determined by direct assays of enzyme activity (65,146). Olwin et al. (133) has shown that the presence of light chains and the non hydrolysable analogue of ATP, AppNHP, reduced the dissociation constant of calmodulin for skeletal MLCK by a factor of 5. Thus a Kd of 9 nM compares favorably with what should be expected in the absence of substrates. MIANS-CaM binds to caldesmon with an affinity that is 25 times lower than that for MLCK. At present the physiological releveance of the interaction between caldesmon and calmodulin is not known. Kd determinations much below 10 nM are difficult with MIANS because of low fluorescence relative to background. The affinity of calcinurin for MIANS-CaM could not be accurately determined for this reason. Labeling of spinach calmodulin with rhodamine-X maleimide allowed Kd determinations for calcineurin in the low nanomolar range. The Kd of 1.5 nM that we report is similar to the estimate of 3 nM reported recently by Putkey et al. (100), and very similar to the K0.5 of 2.5 nM that we observe for cGMP phosphodiesterase with MIANS-CaM (Figure 22).

Titrations of MIANS-CaM with melittin, prenylamine and calmidazolium indicate that the binding of these compounds to calmodulin does not alter MIANS fluorescence. This is in contrast to the findings with diethylaminonapthalene-1-sulfonic acid-calmodulin,
which shows similar fluorescence changes with the binding of proteins (146), small peptides, (146) and calmodulin antagonist drugs (77). MIANS calmodulin appears to be sensitive primarily to its interactions with calmodulin's target proteins.

The fluorescence of the four labeled calmodulins shown in Fig 23 covers the entire visible range. These fluorescent calmodulins should prove particularly useful for the microscopic localization of calmodulin target proteins in cells.

MIANS-CaM and rhodamine X-CaM should be especially useful for stop flow studies, which generally cannot use direct assays. Previous studies with stop flow have indicated that the reactions are generally too fast to determine association rate constants when intrinsic fluorescence is used to monitor rate constants (28). The use of these fluorescently labeled CaMs should allow determinations with lower enzyme concentrations so that association rates for calmodulin and its target proteins can be measured. This type of study should be useful in determining the temporal progression of calmodulin activation and regulation of cellular events.
Figure 18. Reaction of MIANS with spinach and bovine brain calmodulin. Fluorescence was monitored at 440 nM with excitation at 320 nM. Spinach or brain calmodulins (3 μM) were added as indicated to MIANS (10 μM) in 10 mM MOPS (pH 7.0), 90 mM KCl, 2 mM EGTA. Inset shows the fluorescence emission spectra of (1) MIANS alone, (2) MIANS incubated 120 min with spinach calmodulin, and (3) MIANS incubated 120 min with bovine brain calmodulin. Subsequent addition of spinach CaM to the brain CaM-MIANS solution resulted in reactivity as indicated.
Figure 19. Calcium titrations of MIANS labeled and native spinach calmodulin. Buffer was 200 mM MOPS (pH 7.0), 90 mM KCl, and 2 mM EGTA. MIANS fluorescence (△ - △) calmodulin excitation 320 nM, emission 440 nM; and (○ - ○) tyrosine fluorescence in unlabeled calmodulin, excitation 275 nM, emission 310 nM are shown as a function of free calcium.
Figure 20. Titrations of MIANS calmodulin (10 nM) with calcinurin (O), MLCK (■) and 50 nM MIANS-CaM with caldesmon (▲). Concentrations represent total target protein added. Buffer was 10 mM MOPS, 90 mM KCl and 1 mM CaCl₂, pH 7.0.
Figure 21. Scatchard analysis of MLCK and caldesmon binding to MIANS calmodulin. Total MIANS-CaM was 5 nM with MLCK (A) and 50 nM with caldesmon (B). The correlation coefficients for MLCK and caldesmon were .979 and .999, respectively. 4A inset shows fluorescence emission spectra of buffer, (a) 10 nM MIANS-CaM (b), and 10 nM MIANS-CaM, 40 nM MLCK (c). ΔF represents the increase relative to that of MIANS-CaM, i.e. ΔF=1 is 100% increase in fluorescence.
Figure 22. Activation of cGMP phosphodiesterase by unlabeled (♦) or MIANS labeled (●) spinach calmodulin. Reaction was done in 10 mM MOPS (pH 7.0) 90 mM KCl, 5 mM MgCl₂, and 1 mM CaCl₂, containing 8 μM 2′(N methyl anthraniloyl) cGMP. Both calmodulins produced a 24 fold activation.
Figure 23. Fluorescence emission spectra of (a) DMSM (b) MIANS (c) acrylodan or (d) rhodamine X-maleimide labeled spinach calmodulin. Excitation was 320 nm for DMSM and MIANS, 390 nm for acrylodan and 550 nm for rhodamine-X-maleimide. Spectra were done with 50 nM of each labeled CaM and spectra were corrected for Raman and background fluorescence.
General Discussion

We have examined the binding of felodipine to calmodulin and shown that there are two cooperative binding sites for felodipine on calmodulin. Other drugs, including calmidazolium, prenylamine, and diltiazem, bind to the same sites and are capable of enhancing felodipine binding at low felodipine concentrations. Various metals, including zinc, cadmium, lanthanum, terbium, and lead bind to the conformer that has a high affinity for felodipine and enhance felodipine binding. The effect of metals is distinct from that of drugs in that the drugs bind to the other felodipine site (i.e. a cooperative site), whereas the metals bind to an allosteric site. Metals enhance felodipine binding even at high felodipine concentrations whereas the competitive ligands inhibit binding at high felodipine concentrations (see figure 3 vs figure 11 for comparison). Those metals which bind to a different conformation (i.e. copper and mercury), either at the same site as the potentiating metals or one that is mutually exclusive, reverse the effects of potentiating metals.

Recently, an analogous type of enhancement of calmodulin binding to cyclic nucleotide phosphodiesterase (PDE) was reported, in this case by an antibody which binds to calmodulin only in the presence of calcium and which binds to calmodulin even more tightly (60 fold) in the presence of PDE and calcium (147). The antibody was able to enhance calmodulin's ability to activate PDE by about 60 fold.
also, as would be expected on the theory of energy coupling (148). This implies that both PDE and the antibody bind to the same conformation, exactly analogous to what we see with potentiating metals and felodipine. The binding enhancement by PDE was relatively specific; it also occurred with phosphorylase kinase, but calmodulin binding to the antibody was inhibited by calcineurin and MLCK. The drug trifluoperazine enhanced antibody affinity for calmodulin while the drug 48/80 reduced the affinity indicating that different drugs, like different target proteins prefer different calmodulin conformations.

The simplest explanation for the enhancement of antibody affinity by the calmodulin binding proteins is that the CaM-target protein complex is a multi subunit complex. For PDE the complex contains two calmodulin molecules (i.e. PDE$_2$-CaM$_2$) and can thus present two calmodulin molecules to the two binding sites on the antibody simultaneously. Phosphorylase kinase is also a multi subunit complex. The authors dismissed this type of crosslinking by PDE as an explanation, since high concentrations of PDE did not inhibit binding as they expected. However, since PDE interacts cooperatively with calmodulin, all complexes should be either PDE$_2$-CaM$_2$ or PDE$_2$, and PDE$_2$-CaM$_1$ should never exist even at high concentrations of PDE.

Could dimerization also account for the cooperativity of felodipine binding? Analyses of ligand mediated dimerization show
that such a process can exhibit cooperativity (149). Wyman et al. (106), have shown that monomers cannot exhibit stereospecific cooperative binding. Thus for the cooperativity observed with felodipine binding to calmodulin to be stereospecific, and thus applicable to real protein-protein interactions the cooperativity must be via ligand mediated dimerization. Calcium enhanced dimerization of troponin C has been shown to occur, so a similar process may occur with calmodulin. Dimerization would be expected to be enhanced by binding of additional cations and lowering of pH, since both processes would reduce charge repulsion, since calmodulin is highly negatively charged at neutral pH.

The other possibility is that the two felodipine binding sites represent two hydrophobic interaction sites (not stereospecific) which interact simultaneously with the target protein and that the interaction occurs with a high degree of symmetry, since cooperativity is symmetry dependent. This symmetrical interaction could be useful in the process of activation of a target protein, since it could impart some symmetry to the CaM-protein complex, and this symmetry could be necessary for target enzyme activity. This second possibility does not rule out dimerization also occurring, and indeed dimerization may be important in activating only some target proteins. If true, then regulating the degree of dimerization would allow selective
activation of a group of target proteins.

In order to monitor calmodulin interactions with target proteins, it is necessary to be able to work in the low nanomolar range, since most target proteins bind with low nanomolar affinity (see table I). To this end, several highly fluorescent derivatives of calmodulin have been synthesized. These fluorescent calmodulins activate PDE as well as unmodified calmodulin. Spinach calmodulin was used since it contains a single cysteine, and cysteine can be modified in a highly selective manner. These fluorescent calmodulins should be useful in probing for allosteric interactions between calmodulin and its target proteins, which the cell may use to specifically activate a group of target proteins under a particular set of conditions. It should also be useful in investigating the aforementioned antibody, and its interaction with calmodulin-target protein complexes.
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