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Kinetics of calmodulin binding to its smooth muscle target proteins

Kasturi, Rama, Ph.D.
The Ohio State University, 1991
KINETICS OF CALMODULIN BINDING
TO ITS SMOOTH MUSCLE TARGET PROTEINS

DISSERTATION

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

by

Rama Kasturi, B.S.

* * * * * *

The Ohio State University

1991

Dissertation Committee

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The Ohio State University
Biophysics Program
To My Grandparents, Parents and Sister
ACKNOWLEDGEMENTS

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Finally, I wish to thank my parents, Prema and Kasturi, without whom none of this would have been possible.
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Major Field: Biophysics
B. RESULTS ................................ 53
C. DISCUSSION .......................... 68

IV. LIST OF REFERENCES ............... 82
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. Cellular processes mediated by calmodulin</td>
<td>9</td>
</tr>
<tr>
<td>II. Summary of the kinetics of calmodulin binding to its target proteins in smooth muscle</td>
<td>71</td>
</tr>
<tr>
<td>III. Summary of the rates of calcium chelator-induced disruption of calmodulin-target protein complexes</td>
<td>73</td>
</tr>
<tr>
<td>IV. Summary of the rates of calcium dissociation from calmodulin-target protein and calmodulin-peptide complexes</td>
<td>78</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Calcium homeostasis</td>
</tr>
<tr>
<td>2</td>
<td>Calcium and calmodulin-mediated events in smooth muscle</td>
</tr>
<tr>
<td>3</td>
<td>Absorption and emission processes</td>
</tr>
<tr>
<td>4</td>
<td>Some commonly used fluorescent probes</td>
</tr>
<tr>
<td>5</td>
<td>Calmodulin-dependence of myosin light chain kinase activation</td>
</tr>
<tr>
<td>6</td>
<td>Calcium-dependence of myosin light chain kinase activation</td>
</tr>
<tr>
<td>7</td>
<td>Interaction of smooth muscle target proteins with fluorescently-labeled wheat calmodulin</td>
</tr>
<tr>
<td>8</td>
<td>Kinetics of myosin light chain kinase binding to calmodulin</td>
</tr>
<tr>
<td>9</td>
<td>Kinetics of caldesmon binding to calmodulin</td>
</tr>
<tr>
<td>10</td>
<td>Kinetics of calmodulin-target protein complex disruption by a calcium chelator</td>
</tr>
<tr>
<td>11</td>
<td>Kinetics of calcium dissociation from calmodulin and calmodulin-target protein complexes</td>
</tr>
<tr>
<td>12</td>
<td>Kinetics of the EGTA-induced decrease in the intrinsic tyrosine fluorescence of wheat calmodulin</td>
</tr>
</tbody>
</table>
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-TM</td>
<td>Thin filament actin-tropomyosin domain in smooth muscle</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>ANS</td>
<td>Anilino naphthalene sulfonate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAPTA</td>
<td>1,2 bis (2-aminophenoxy)ethane-N,N,N′,N′ tetraacetic acid</td>
</tr>
<tr>
<td>[Ca$^{2+}$]_i</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>CaD</td>
<td>Caldesmon</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaM-MIANS</td>
<td>Wheat CaM covalently labeled with MIANS</td>
</tr>
<tr>
<td>CaM Kinase</td>
<td>Type II Ca$^{2+}$/CaM dependent kinase</td>
</tr>
<tr>
<td>CaN</td>
<td>Calcineurin</td>
</tr>
<tr>
<td>CaP</td>
<td>Calponin</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DANS</td>
<td>Dimethyl-aminonaphthalene-1-sulfonyle chloride</td>
</tr>
<tr>
<td>Dans-CaM</td>
<td>CaM covalently labeled with DANS</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylaminoethyl</td>
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x
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine N,N,N',N'-tetra acetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol bis-(β-aminoethylether) N,N,N',N'-tetra acetic acid</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>GMP</td>
<td>Guanosine monophosphate</td>
</tr>
<tr>
<td>G-protein</td>
<td>Guanine nucleotide-regulated protein</td>
</tr>
<tr>
<td>IAANS</td>
<td>2-[4′-iodoacetamidoanilino]-naphthalene-6-sulfonic acid</td>
</tr>
<tr>
<td>IAANS-CaM</td>
<td>CaM covalently labeled with IAANS</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>K$_d$</td>
<td>Equilibrium dissociation constant</td>
</tr>
<tr>
<td>k$_{on}$</td>
<td>Association rate constant</td>
</tr>
<tr>
<td>k$_{off}$</td>
<td>Dissociation rate constant</td>
</tr>
<tr>
<td>MIANS</td>
<td>2-(4′-maleimidylanilino)-naphthalene-6-sulfonic acid</td>
</tr>
<tr>
<td>MLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propane-sulfonic acid</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>PDE</td>
<td>3′,5′- cyclic nucleotide phosphodiesterase</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>Phosphatidyl inositol bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Cyclic-AMP dependent protein kinase A</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>P-MLCK</td>
<td>MLCK phosphorylated by PKA</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>QF</td>
<td>Fluorescence quantum yield</td>
</tr>
<tr>
<td>Quin 2</td>
<td>2-[(2-bis-[carboxymethyl]amino-5-methylphenoxy)methyl-6-methoxy-8 bis[carboxymethyl] aminoquinoline</td>
</tr>
<tr>
<td>ROC</td>
<td>Receptor-operated calcium channel</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
</tr>
<tr>
<td>TFP</td>
<td>Trifluoperazine</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I</td>
</tr>
<tr>
<td>TNS</td>
<td>Toluidinyl naphthalene sulfonate</td>
</tr>
<tr>
<td>VOC</td>
<td>Voltage-operated calcium channel</td>
</tr>
</tbody>
</table>
CHAPTER I
INTRODUCTION

A. Historical background of the Ca\textsuperscript{2+} signal

In 1883, Sidney Ringer demonstrated that the contractile activity of frog heart could be maintained \textit{in vitro} in the presence of a Ca\textsuperscript{2+}-containing medium (1). Subsequently, extracellular Ca\textsuperscript{2+} was observed to play a major role in skeletal (2) and smooth muscle (3) contractility and in neuromuscular transmission (4). These observations were the first to implicate extracellular Ca\textsuperscript{2+} as an important modulator of biological processes.

In 1942, Bailey (5) and Needham (6) demonstrated that Ca\textsuperscript{2+} was necessary to activate the acto-myosin ATPase activity of muscle. In 1947, Heilbrunn and Wiercinski (7) showed that injection of Ca\textsuperscript{2+} into muscle fibers caused contraction. Subsequent work by Kielley and Meyerhof (8,9) and Marsh (10,11) led to the discovery that a granular ATPase fraction was responsible for relaxation in skeletal muscle. Bozler (12), Watanabe (13) and Ebashi (14) showed that the
Ca\textsuperscript{2+} chelators EDTA and EGTA, mimicked the effect of this granular ATPase and relaxed glycerinated muscle fiber. Concurrently, Ebashi (15,16) and Hasselbach and Makinose (17) demonstrated that the granular ATPase could accumulate Ca\textsuperscript{2+} in the presence of ATP. Electron microscopic studies by Nagai et al. (18) and Ebashi (15) revealed that the granular ATPase was derived from the endoplasmic (sarcoplasmic) reticulum of muscle. On the basis of these findings, it was established that the contraction-relaxation cycle in skeletal muscle was regulated by Ca\textsuperscript{2+}, with the sarcoplasmic reticulum of muscle exerting its effect by sequestering Ca\textsuperscript{2+} from the contractile system.

The discovery of the troponin-tropomyosin complex by Ebashi and his colleagues (19,20), established troponin as the Ca\textsuperscript{2+}-receptor linked to thin filaments in skeletal muscle. They proposed that binding of Ca\textsuperscript{2+} to troponin produced conformational changes in F-actin that resulted in force-generating acto-myosin interactions. Thus, Ca\textsuperscript{2+} was established as the common link between excitation at the muscle membrane produced by physiological stimuli and the resultant contraction i.e., excitation-contraction coupling.
The discovery in 1964 by Meyer and his co-workers (21) that Ca\textsuperscript{2+} was required for phosphorylation of phosphorylase kinase was followed by the observation of Ozawa and his co-workers in 1967 (22) that the Ca\textsuperscript{2+} concentrations required for the activation of phosphorylase kinase were similar to those required for activation of contractile elements in skeletal muscle. This observation helped to establish Ca\textsuperscript{2+} as a mediator of excitation-metabolism coupling.

The second messenger concept proposed by Sutherland et al. in 1968 (23) was used by Rasmussen (24) to describe how signals (messages) arriving at the membrane surface were coupled to contractile, metabolic and secretory events through a transient increase in intracellular Ca\textsuperscript{2+} (~10\textsuperscript{-7} M to 10\textsuperscript{-6} M), the second messenger.

B. Ca\textsuperscript{2+} as a second messenger

Any chemical designed to function as an intracellular messenger must experience large fluctuations in concentration around the targets of messenger function. The concentration of calcium in the extracellular space is ~3 mM, with half of this calcium in the ionized form. The intracellular free
Ca\(^{2+}\) concentration is maintained at sub-micromolar (10\(^{-7}\) M) levels. This results in a 10,000-fold gradient of Ca\(^{2+}\) across the plasma membrane and, consequently, even minor changes in the Ca\(^{2+}\) permeability of the plasma membrane induced by primary messengers (hormones and neurotransmitters) produce significant changes in its cytosolic concentration. The resulting influx of Ca\(^{2+}\) in response to physiological stimuli acting upon the plasma membrane is detected as a message (signal) and translated into metabolic or contractile responses.

C. Regulation of intracellular Ca\(^{2+}\)

Cells cannot tolerate a high internal concentration of Ca\(^{2+}\) i.e. [Ca\(^{2+}\)]\(_i\) because it combines with inorganic phosphate found intracellularly, sequestering it as insoluble hydroxyapatite. The plasma membrane of the cell plays a major role in the regulation of [Ca\(^{2+}\)]\(_i\) levels through voltage-operated (VOC) and receptor-operated Ca\(^{2+}\) channels (ROC) (see Figure 1). Hydrolysis of phosphatidyl inositol bisphosphate (PIP\(_2\)), by receptor-mediated G-protein activation of membrane phospholipase C (PLC), produces IP\(_3\) which results in the release of endoplasmic
Figure 1  **Calcium homeostasis.** A rise in intracellular Ca\(^{2+}\), [Ca\(^{2+}\)]\(_i\), occurs by the entry of Ca\(^{2+}\) through plasma membrane voltage-operated and receptor-operated Ca\(^{2+}\) channels and by the agonist-induced (hormones and neurotransmitters) release of Ca\(^{2+}\) from endoplasmic (sarcoplasmic) reticular stores. The binding of agonists to their plasma-membrane receptors results in the activation of a G-protein that stimulates membrane phospholipase C (PLC) hydrolysis of phosphatidyl inositol bisphosphate (PIP\(_2\)). PIP\(_2\) hydrolysis results in the formation of inositol trisphosphate (IP\(_3\)). IP\(_3\) releases Ca\(^{2+}\) from the endoplasmic reticulum. G-protein activation of some ROC’s results in an influx of extracellular Ca\(^{2+}\). [Ca\(^{2+}\)]\(_i\) is actively extruded from the cell or sequestered in the endoplasmic reticulum by plasma membrane and endoplasmic reticular Ca\(^{2+}\)-ATPases. The reversible Na\(^+\)/Ca\(^{2+}\) antiporters also play a role in the regulation of [Ca\(^{2+}\)]\(_i\). The rise in [Ca\(^{2+}\)]\(_i\) results in the activation of Ca\(^{2+}\)-dependent proteins e.g. calmodulin, that regulate Ca\(^{2+}\)-dependent cellular events.
(sarcoplasmic) reticular Ca\textsuperscript{2+} stores. The plasma membrane also contains high affinity, low capacity Ca\textsuperscript{2+}-ATPase pumps that actively extrude Ca\textsuperscript{2+} from the cytosol and low affinity, high capacity, reversible Na\textsuperscript{+}/Ca\textsuperscript{2+} antiporters that transport Ca\textsuperscript{2+} to the extracellular space using the gradient of Na\textsuperscript{+} that exists across the cell membrane (see (25) for review).

Certain internal organelles play an important role in regulating [Ca\textsuperscript{2+}]\textsubscript{i}. The endoplasmic reticulum is a high affinity, high capacity system with separate Ca\textsuperscript{2+} uptake (Ca\textsuperscript{2+}-ATPase) and release mechanisms for controlling cytosolic Ca\textsuperscript{2+}. Mitochondria serve as high capacity, low-affinity intracellular Ca\textsuperscript{2+} regulators playing a regulatory role only when intracellular Ca\textsuperscript{2+} concentrations become very high (> 5 μM).

All cells also contain a variety of Ca\textsuperscript{2+}-binding proteins that buffer increases in intracellular Ca\textsuperscript{2+} and process the Ca\textsuperscript{2+} signal. However, the major role of the Ca\textsuperscript{2+}-modulated proteins is in the processing of the Ca\textsuperscript{2+} signal. These proteins include the multifunctional calmodulin (CaM) and the specialized (tissue-specific) Ca\textsuperscript{2+}-binding proteins such as troponin C (TnC), intestinal Ca\textsuperscript{2+}-binding protein,
S-100 protein and the regulatory light chains of myosin.

D. Calmodulin in calcium signal transduction

Since its discovery in 1970 by Cheung (26) and Kakiuchi and Yamazaki (27), CaM has emerged as the universal transducer of the Ca$^{2+}$ signal. CaM is found in nearly all organelles of all eukaryotic cells with a high degree of conservation of its primary structure. This is consistent with its role as a mediator of multiple, fundamental, Ca$^{2+}$-controlled physiological processes. Unlike TnC which specifically mediates skeletal and cardiac muscle contraction, CaM mediates Ca$^{2+}$-regulation of a large number of proteins and enzymes directly, through interaction with target enzymes or indirectly, via activation of specific kinases, triggering biochemical responses such as energy metabolism, cell motility, cytoskeletal assembly, smooth muscle contraction, DNA replication and exocytosis (see Table I).

CaM is a water soluble, cytosolic protein of molecular weight 16,680 Da (see (28) for review). Its cellular level in vertebrates varies from 20-60 µM in brain, testis, gizzard and uterus to less than 10 µM in
Table I  Cellular processes mediated by calmodulin.

<table>
<thead>
<tr>
<th>PROTEIN/ENZYME</th>
<th>Biochemical process mediated by Ca^{2+} and CaM activation of Protein/Enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclic nucleotide phosphodiesterase</td>
<td>control of c-AMP, c-GMP levels</td>
</tr>
<tr>
<td>Adenylate cyclase</td>
<td>control of c-AMP levels</td>
</tr>
<tr>
<td>Smooth muscle MLCK</td>
<td>smooth muscle contraction</td>
</tr>
<tr>
<td>Phosphorylase kinase</td>
<td>glycogenolysis</td>
</tr>
<tr>
<td>Calmodulin Kinase II</td>
<td>neurosecretion</td>
</tr>
<tr>
<td>Calcineurin</td>
<td>cellular dephosphorylation</td>
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<td>Calcium-ATPase</td>
<td>calcium homeostasis</td>
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<td>Caldesmon</td>
<td>smooth muscle contraction</td>
</tr>
<tr>
<td>Calponin</td>
<td>smooth muscle contraction</td>
</tr>
<tr>
<td>Phosphofructokinase</td>
<td>glycolysis</td>
</tr>
<tr>
<td>NAD-kinase</td>
<td>cellular metabolism in plants</td>
</tr>
</tbody>
</table>
liver and heart and skeletal muscle. Vertebrate CaM is a heat stable, acidic (pI = 4.1) 148-residue protein that contains no tryptophan, cysteine, or carbohydrate. Plant CaM contains a free sulfhydryl group as a result of the substitution of Cys 26 (29) or Cys 27 (30) for Thr 26 in vertebrate CaM.

CaM's impressive ability to recognize Ca$^{2+}$ specifically over other intracellular ions present in much larger concentrations (intracellular concentrations of Mg$^{2+}$ and K$^+$ are 1-5 mM and 100 mM, respectively) lies in the unique molecular architecture of its Ca$^{2+}$-binding sites. Structurally, CaM belongs to the family of E-F hand proteins as defined by Kretsinger (31). These E-F hands consist of a Ca$^{2+}$-binding loop of 12 amino acids flanked by two regions of nearly perpendicular alpha-helix. The elucidation of the primary structure of CaM by Watterson et al. (32) predicted that CaM contained four helix-loop-helix repeats (E-F hands). The four Ca$^{2+}$-binding domains (E-F hands) in CaM are homologous, with greater homology seen between domains I and III than between II and IV.

The elucidation of the crystal structure of CaM by Babu et al. (33) established that in the presence of Ca$^{2+}$, CaM is a dumbbell-shaped molecule about 65 Å
long with N- and C-terminal halves with approximate dimensions of 25 X 20 X 20 Å, each containing a pair of Ca\textsuperscript{2+}-binding domains. These Ca\textsuperscript{2+}-binding domains are numbered I-IV starting from the N-terminus of the protein. The N- and C-terminals of CaM are connected by a highly negatively charged, 8-turn alpha-helix about 20 Å in length. The molecule is ~63% alpha-helical with the only beta-sheet structure occurring between each of the two pairs of Ca\textsuperscript{2+}-binding loops. The ligands coordinating to Ca\textsuperscript{2+} include oxygen atoms from the side chains of Asp and Glu residues, Ser and Thr hydroxyl groups, carbonyl groups from the protein backbone and water molecules. The coordination sphere is approximately octahedral.

Ca\textsuperscript{2+} binds to CaM producing conformational changes that result in an increase in CaM’s intrinsic tyrosine fluorescence and an increase in its alpha-helical content (34), changes in its UV absorption (35) and NMR spectra (36) and exposure of hydrophobic regions on the N- and C-terminal lobes of CaM (37-39).

X-ray crystallographic studies of CaM (33) show the presence of a hydrophobic cleft in each half of the molecule consisting of the aromatic rings of Phe 19, Phe 65 and Phe 68 in the N-terminal half and Phe 89,
Tyr 138 and Phe 141 in the C-terminal half. These hydrophobic clefts border the helical interface of each pair of Ca$^{2+}$-binding sites which are linked by beta-sheet structure. The proximity of these hydrophobic clefts to the Ca$^{2+}$-binding sites allows for cooperative interactions between the hydrophobic sites and the Ca$^{2+}$-binding sites in each half of the molecule.

The rates of acetylation of lysines 75 and 148 which extend over the hydrophobic clefts associated with each half of the CaM molecule exhibit a large and specific decrease when either calcineurin (CaN) or myosin light chain kinase (MLCK) is complexed with CaM (40,41). NMR studies suggest that these hydrophobic clefts may be involved in the binding of CaM to target proteins (42), CaM-antagonist drugs (43) and CaM-antagonist peptides (44). Exposure of hydrophobic sites on CaM has been observed by the Ca$^{2+}$-dependent binding of the hydrophobic, fluorescent molecules anilino naphthalene sulfonate (ANS), toluidinyl naphthalene sulfonate (TNS) and felodipine (a Ca$^{2+}$ channel blocker) (37-39) to CaM. The Ca$^{2+}$-dependence of the binding of these molecules to CaM correlates well with the Ca$^{2+}$-dependence of activation of CaM's target proteins (45,46). Also, the binding of
hydrophobic CaM-antagonist drugs to these N- and C-terminal sites on CaM results in the complete inhibition of target-protein activation by CaM (47). In addition, there is evidence for cooperativity between the N- and C-terminal hydrophobic clefts upon binding of CaM-antagonist drugs (39). These data suggest that the binding of Ca\textsuperscript{2+} to CaM, results in the exposure of allosterically-regulated hydrophobic sites on the N- and C-terminal lobes of CaM, which are necessary for the binding of CaM-antagonist drugs, peptides and activation of target proteins.

Babu et al. (33) have suggested that the central helix connecting the N- and C-terminal Ca\textsuperscript{2+}-binding domains is most likely buried in the absence of Ca\textsuperscript{2+} and exposed upon Ca\textsuperscript{2+}-binding. Small angle X-ray scattering experiments have indicated that Ca\textsuperscript{2+} produces an elongation of CaM from 58 to 62 Å (48). All of these results are consistent with a Ca\textsuperscript{2+}-induced conformational change in the central helix. It has been shown that tryptic cleavage of CaM at Lys 77 in the central helix occurs only in the presence of Ca\textsuperscript{2+} (49). Carboxymethylation of CaM's methionine residues occurs at Met 71, 72 and 76 only in the presence of Ca\textsuperscript{2+} resulting in the inhibition of the
CaM–phosphodiesterase (PDE) interaction (50). Spectroscopic studies indicate that the Ca$^{2+}$-dependent tryptic cleavage at Lys 77 does not appreciably alter the conformation or the ligand binding properties of the two halves (51–53). Yet, neither alone nor in combination, fragments 1–77 and 78–148 cannot activate skeletal muscle MLCK, PDE or CaN (54–55). This suggests that both halves of the CaM molecule interact with a target protein CaM-binding domain and that the central helix is necessary for Ca$^{2+}$-dependent activation of target enzymes by CaM.

Low angle X-ray scattering data (56) for CaM complexed with the CaM-antagonist peptide, melittin, or target protein, skeletal muscle MLCK, indicate that the individual lobes of CaM are considerably closer together in their presence than in the crystal structure of Ca$^{2+}$-saturated CaM. A mutant vertebrate CaM has been shown to retain high activity toward skeletal muscle MLCK activation even when the central helix is cut and the N- and C-terminals are covalently cross-linked (57). The central helix of CaM appears, therefore, to serve as a flexible tether allowing the two halves of the CaM molecule sufficient mobility to form a variety of specific target protein or peptide
binding sites (58). This results in CaM binding its target proteins with high affinity and broad specificity.

Recent studies using X-ray crystallography, site-directed mutagenesis and fluorescent CaM-binding peptides have shown that the plurality of CaM’s interactions with its target proteins lies in its ability to recognize positively charged (basic), amphiphilic, alpha-helical peptides independent of their precise amino-acid sequences (see (58) for review). Segments capable of adopting this configuration are frequently found in the sequences of CaM-binding proteins (59). The hydrophobic clefts of the N- and C-terminal domains of CaM are flanked by highly negatively charged regions that offer excellent binding sites for these basic, amphiphilic, alpha-helical, CaM-binding domains of target proteins and peptides (60,61). The binding of CaM to its target proteins probably involves both hydrophobic and electrostatic interactions.

E. Ca\(^{2+}\) binding to CaM

CaM has four Ca\(^{2+}\) binding sites. Direct binding studies of the binding of Ca\(^{2+}\) to CaM have demonstrated
that the affinities of Ca$^{2+}$ for CaM are in the range $2 \times 10^{-6}$ to $1 \times 10^{-4}$ M (62-64). The rates of Ca$^{2+}$ dissociation from CaM, measured by stopped-flow using the fluorescent Ca$^{2+}$ indicator Quin 2 (65) and the fluorescent probes ANS (66) and TNS (67), are 240 - 600 s$^{-1}$ and 10 - 30 s$^{-1}$ suggesting the presence of high and low affinity Ca$^{2+}$-binding sites on CaM. These Ca$^{2+}$ dissociation rates are similar to the fast (600-1000 s$^{-1}$) and slow (10-30 s$^{-1}$) Ca$^{2+}$ exchange rates measured using $^{43}$Ca NMR and $^1$H NMR (36,68,69). The slow exchange rates obtained from these NMR data are similar to the Ca$^{2+}$ dissociation rates of 10.4 - 12.6 s$^{-1}$ measured by stopped-flow studies monitoring the changes in tyrosine or dansyl fluorescence following the chelation of Ca$^{2+}$ from the Ca$^{2+}$-CaM complex or the dissociation of Ca$^{2+}$ from the Ca$^{2+}$-CaM complex with the fluorescent Ca$^{2+}$ indicators BAPTA or Quin 2 (64,70,71). These data provide evidence for high and low-affinity Ca$^{2+}$-binding sites on CaM. Studies with tryptic fragments of CaM cleaved at Lys 77 (43,52) have allowed the assignment of the C-terminal Ca$^{2+}$-binding sites III and IV as the high affinity sites and the N-terminal Ca$^{2+}$-binding sites I and II as the low affinity sites.
The question of whether Ca\textsuperscript{2+} binding to CaM is sequential or random has been the subject of much discussion. The stopped-flow and NMR data mentioned above, together with indirect spectroscopic studies of Ca\textsuperscript{2+} binding to CaM using CaM's intrinsic tyrosine fluorescence, circular dichroism and UV spectroscopy (34,35), suggest that Ca\textsuperscript{2+} binding to CaM is a sequential process i.e. Ca\textsuperscript{2+} first binds cooperatively to the high affinity C-terminal Ca\textsuperscript{2+} sites followed by cooperative binding to the low affinity N-terminal sites. A recent study by Linse et al. (72) using the calcium indicator BAPTA has confirmed this sequential, cooperative process of Ca\textsuperscript{2+} binding to CaM.

Burger et al. (73) and Cox et al. (75) have concluded from analyses of equilibrium Ca\textsuperscript{2+}-binding data that at physiological ionic strength, Ca\textsuperscript{2+} binding to CaM is a non-sequential process i.e., the observed, major conformational change in CaM with the binding of 2 moles of Ca\textsuperscript{2+} is the result of Ca\textsuperscript{2+} binding to any two sites in CaM. This conflict between the equilibrium Ca\textsuperscript{2+}-binding data (45,73) and the kinetic and spectroscopic data was addressed by Wang, (74) who showed that the equilibrium binding data could be fit equally well with either a bi-phasic model of Ca\textsuperscript{2+}
binding with cooperativity between each pair of Ca\(^{2+}\)-binding sites or by a model of random binding with no cooperativity. He concluded that the equilibrium dialysis method for determining Ca\(^{2+}\)-binding to CaM was not sensitive enough to allow for a distinction between these two modes of binding.

In addition to the evidence for the cooperativity of Ca\(^{2+}\)-binding within each pair of Ca\(^{2+}\)-binding sites, there is evidence to support the idea of cooperativity between the N- and C-terminal Ca\(^{2+}\) binding sites. Binding of Ca\(^{2+}\) to the N-terminal sites of CaM affects His 107 in the C-terminal half of CaM as detected by \(^{1}\)H NMR (75) and Ca\(^{2+}\) binding to its C-terminal sites affects wheat CaM spin-labeled at Cys 27 (76). A recent study by Beckingham (77) provides new evidence for cooperativity between the N- and C-terminal Ca\(^{2+}\)-binding sites and suggests that the N-terminal Ca\(^{2+}\)-binding sites may be more independent with respect to Ca\(^{2+}\)-binding compared with the C-terminal sites.

The prevailing view is that CaM has a pair of high and low affinity Ca\(^{2+}\)-binding sites located in the C- and N-terminal halves of the molecule, respectively, with positive cooperativity of Ca\(^{2+}\)-binding occurring between each pair of Ca\(^{2+}\)-binding sites. Recent studies
with the calcium indicator BAPTA (72) suggest that the affinity of the C-terminal Ca\(^{2+}\) sites is \(6\)-fold higher than that of the N-terminal Ca\(^{2+}\) sites.

**F. Effect of target protein binding on CaM's calcium affinity**

A general mechanism for the Ca\(^{2+}\)-dependent activation of various target proteins has been described (26,28,78). The reactions involving the activation process are

\[
\text{nCa}^{2+} + \text{CaM (inactive) } \rightleftharpoons \text{Ca}^{2+}\text{n.CaM}^{*} \text{(active)}
\]

\[
\text{Ca}^{2+}\text{n.CaM}^{*} + \text{E(inactive) } \rightleftharpoons \text{Ca}^{2+}\text{n.CaM}^{*}\text{E}^{*} \text{(active)},
\]

where \(n = \text{number of Ca}^{2+}\).

In general, \(\text{Ca}^{2+}_{3}\).\text{CaM}^{*} \text{ or } \text{Ca}^{2+}_{4}\).\text{CaM}^{*} has been shown to be necessary for CaM-activation of target enzymes (45).

Upon interaction with target proteins, CaM acquires strong cooperativity with respect to its Ca\(^{2+}\)-binding (79). This has also been seen with CaM antagonist-drug or peptide binding (80,81), suggesting that in the presence of target protein, peptide or drug, Ca\(^{2+}\)-binding to the N- and C-terminal halves of CaM is no
longer independent. This results in a 7 to 35-fold increase in the Ca$^{2+}$ affinity of CaM upon complex formation with calmidazolium (80), melittin (82), troponin I (83), phosphodiesterase (84) and skeletal muscle MLCK (85).

Stopped flow studies of the Ca$^{2+}$ dissociation rates from CaM-protein, CaM-antagonist drug and CaM-antagonist peptide complexes suggest an increase in the Ca$^{2+}$ affinity of CaM upon complex formation with drugs and peptides. The binding of the CaM-antagonist drug trifluoperazine (TFP) results in a 6.5-fold decrease (7.7 s$^{-1}$ to 1.08 s$^{-1}$) in the rate of Ca$^{2+}$ dissociation from the high affinity (C-terminal) sites of CaM (65). The CaM antagonist-peptide melittin produces an increase in the Ca$^{2+}$ affinity of both the N-terminal and C-terminal Ca$^{2+}$ binding sites (71). The Ca$^{2+}$ dissociation rate from the high affinity sites of CaM was reduced from 13.4 s$^{-1}$ to 1.38 s$^{-1}$ in the presence of melittin (71). Ca$^{2+}$-dissociation from the C-terminal lobe of CaM in the absence of target proteins was observed to occur at 10 s$^{-1}$ and 12 s$^{-1}$, using the fluorescent probe anthroylcholine (86) and the fluorescent Ca$^{2+}$ indicator BAPTA (64), respectively. In the presence of TnI and PDE, Ca$^{2+}$
dissociates from the CaM-TnI and CaM-PDE complexes with rates of $6 \text{ s}^{-1}$ and $4 \text{ s}^{-1}$ (64,86), respectively. This suggests that binding of the TnI and PDE to CaM results in a decreased rate of Ca$^{2+}$ dissociation from CaM. This apparent increase in Ca$^{2+}$ affinity observed in the presence of target proteins, drug or peptide, has been attributed to a reduced Ca$^{2+}$ dissociation rate ($K_d = k_{\text{off}}/k_{\text{on}}$) assuming that the association rate of Ca$^{2+}$ with CaM is not significantly altered in the presence of these target proteins. Ogawa and Tanokura (87) have shown that TFP binds to CaM and produces a 57-fold increase in its Ca$^{2+}$ affinity by producing a dramatic 28.5-fold decrease in the Ca$^{2+}$ dissociation rate without significantly increasing (2-fold) the association rate of Ca$^{2+}$ with CaM.

$^{113}\text{Cd}$ NMR studies by Forsen et al. (51) have shown that TFP decreases the cation exchange rates for CaM. Dalgarno et al. (43) have shown that TFP-binding to the hydrophobic clefts in the vicinity of the N- and C-terminal Ca$^{2+}$-binding domains of CaM results in a relative twisting motion of the proximal alpha-helical segments of the Ca$^{2+}$-binding loops. This TFP-induced motion of the alpha-helices is transmitted to the Ca$^{2+}$-binding loops via the beta-sheet connecting them.
Dalgarino et al. (43) have proposed that this drug-mediated conformational change in CaM's N- and C-terminal Ca\(^{2+}\)-binding domains, inhibits the release of Ca\(^{2+}\) from CaM and is responsible for the apparent increase in the Ca\(^{2+}\) affinity of CaM seen in the presence of TFP (51). It has been suggested that target protein binding to CaM could produce similar effects on CaM's Ca\(^{2+}\)-binding sites resulting in an apparent increased affinity of Ca\(^{2+}\) for CaM (see (88) for review).

In summary, the binding of target protein, peptide or CaM-antagonist drug to the Ca\(^{2+}\)-dependent hydrophobic sites on CaM results in a positive cooperativity of Ca\(^{2+}\) binding to CaM. This results in an increase in CaM's affinity for Ca\(^{2+}\) and allows it to become fully Ca\(^{2+}\)-saturated at physiologically relevant Ca\(^{2+}\) concentrations i.e., 1 X 10\(^{-7}\) - 1 X 10\(^{-6}\) M (80,82-85). Thus, target protein-mediated cooperativity of Ca\(^{2+}\) binding to CaM allows CaM to execute its role as a universal transducer of the transient Ca\(^{2+}\) signal at physiological concentrations of Ca\(^{2+}\).
G. Excitation-contraction (E-C) coupling in smooth muscle cells

Contraction in smooth muscle is triggered by a rise in intracellular calcium concentration ([Ca$^{2+}$]$_i$) from resting values of 80-200 nM to ~1 μM (89). This increase in [Ca$^{2+}$]$_i$ is produced by depolarization-induced or action potential-induced alterations of membrane potential (electro-mechanical coupling) and/or by the action of specific hormones and neurotransmitters that effect increases in [Ca$^{2+}$]$_i$ without altering membrane potential (pharmacomechanical coupling) (90). Electro-mechanical coupling results in the activation of voltage-operated calcium channels (VOC’s) in the sarcolemma and release of Ca$^{2+}$ from sarcoplasmic reticular stores. Hormones and neurotransmitters act at specific membrane receptors (e.g., alpha$_1$-adrenergic, H$_1$-histaminergic and cholinergic) to increase [Ca$^{2+}$]$_i$ by receptor-mediated activation of Ca$^{2+}$ channels (ROC’s) and by inositol trisphosphate (IP$_3$)-induced release of Ca$^{2+}$ from sarcoplasmic reticular stores (see Figure 1).
H. Ca\textsuperscript{2+} and calmodulin mediated events in smooth muscle

The initial rise in [Ca\textsuperscript{2+}]\textsubscript{i} which reaches its peak value in \(-500\) ms, is separated from the generation of force in smooth muscle by a 200-400 ms delay (89). This delay is generally ascribed to regulatory events that must precede the formation of cycling acto-myosin cross-bridges that result in the development of muscle force (89). CaM is present in smooth muscle at a concentration of 25-40 \(\mu\text{M}\) (91,92) and has been shown to be essential for smooth muscle contraction (93). The CaM-binding proteins in the contractile apparatus of smooth muscle include myosin light chain kinase (MLCK), caldesmon (CaD) and calponin (CaP) (see Figure 2). The Ca\textsuperscript{2+} and CaM-dependent activation of MLCK is essential for the generation of force in smooth muscle (93). The Ca\textsuperscript{2+}-dependent binding of CaM to CaD and CaP has been suggested to play a role in the initiation and maintenance of force in smooth muscle (94,95).

Myosin light chain kinases have been found in a variety of vertebrate and mammalian muscles and non-muscle tissues (96). Avian gizzard MLCK has been used as a prototype for smooth muscle kinases. Chicken gizzard MLCK is a monomeric, asymmetrical protein with
Figure 2  Calcium and calmodulin-mediated events in smooth muscle. A rise in intracellular $\text{Ca}^{2+}$, results in the activation of CaM. This results in the generation and maintenance of force in smooth muscle by the CaM-dependent regulation of its target proteins, myosin light chain kinase (MLCK), caldesmon (CaD) and calponin (CaP).
\[
Ca^{2+} + CaM \leftrightarrow Ca^{2+}CaM\text{ (active)}
\]

**Figure 2**
a molecular weight of 130,000 Da (97,98). Its cellular concentration in chicken gizzard is reported to be ~1-4 μM (97,99). The exact location of MLCK in the contractile apparatus is still a matter of controversy. Sellers and Pato (100) have suggested that MLCK is predominantly associated with the thin filament (actin), while Sobue et al. have suggested that in the presence of Ca²⁺ and CaM, MLCK is localized on the thick filament (myosin) (101).

Following a rise in [Ca²⁺]ᵢ, Ca²⁺.CaM associates with and activates MLCK, resulting in the phosphorylation of the 20,000 Da myosin light chains (MLC’s). Phosphorylated myosin then interacts with actin to form cycling cross bridges that generate force in smooth muscle by the hydrolysis of ATP (see (102) for review). This is a thick filament-regulated mechanism of force generation. Dephosphorylation of MLC’s occurs by the action of myosin light chain phosphatase. While phosphorylation of MLC’s appears to be essential for the generation of force, dephosphorylation of MLC’s does not always correlate with a decrease in force (103,104).

Caldesmon (CaD) is found in a variety of muscle and non-muscle cells (105). Chicken gizzard CaD is a
highly asymmetric, rod-shaped protein of molecular weight 93,000 Da (106) and may exist in solution as a monomer (107) or dimer (108). Its cellular concentration in chicken gizzard is estimated to be 4-10 μM (99,109). CaD is closely associated with the thin filament actin-tropomyosin (A-TM) domain in smooth muscle. The ratio of CaD to actin in smooth muscle has been reported to be as low as 1:100 (107) or as high as 1:20 (110). It has been proposed that in the absence of Ca²⁺ and CaM, CaD binds to actin-tropomyosin thereby inhibiting cross bridge formation between actin and phosphorylated myosin (109). Association of CaD with Ca²⁺.CaM has been suggested to play a role in the disinhibition of the A-TM domain (109). The disinhibition of the A-TM-CaD domain by Ca²⁺.CaM could play a role in the generation of force in smooth muscle (see (111) for review).

Calponin (CaP) has recently been purified from chicken gizzard (112) and bovine aorta (113). It is a CaM binding protein of molecular weight 34,000 Da. CaP is present in chicken gizzard at a concentration of ~80 μM (113). Electron-microscopic studies have shown that CaP is located on the thin filament A-TM domain (114,115) and is present in a ratio of CaP to actin of
1:7 (95,113). CaP binds actin and tropomyosin in a Ca\textsuperscript{2+}-independent manner, with high affinity (K\textsubscript{d} = 50 nM) and inhibits the interaction of actin with phosphorylated myosin. The binding of CaM to CaP has been suggested to relieve CaP's inhibition of the A-TM domain (95,113). Phosphorylation of CaP by the Ca\textsuperscript{2+}- and phospholipid-dependent protein kinase C (PKC) and the type II Ca\textsuperscript{2+}- and CaM-dependent kinase (CaM Kinase II) relieves its inhibition of the acto-myosin interaction (116). Phosphorylation of CaP by PKC is inhibited by Ca\textsuperscript{2+}.CaM suggesting that the Ca\textsuperscript{2+}-dependent interaction of CaM with CaP may regulate PKC phosphorylation of CaP and smooth muscle contraction (116).

I. Mechanisms regulating smooth muscle contraction and relaxation

Contraction in smooth muscle involves two stages

(a) Force development (phase I)
(b) Force maintenance (phase II)

It is generally accepted that development of force in smooth muscle is regulated by the Ca\textsuperscript{2+}.CaM-dependent phosphorylation of MLC's by MLCK. In addition, the
thin filament CaM-binding proteins CaD and CaP may contribute to force development as described above.

Recent studies have suggested the existence of a separate, relatively \([\text{Ca}^{2+}]_i\) independent mechanism for the regulation of smooth muscle contraction involving PKC (117). In the absence of any significant change in \([\text{Ca}^{2+}]_i\), agonist-mediated (e.g., prostaglandin \(F_{2\alpha}\)) activation of PKC by diacylglycerol (DAG) and resting \([\text{Ca}^{2+}]_i\) has been suggested to initiate the development and maintenance of force in smooth muscle, presumably by the phosphorylation of one or more proteins (117).

Thus, regulation of \([\text{Ca}^{2+}]_i\)-dependent contraction in smooth muscle occurs primarily by the \(\text{Ca}^{2+}.\text{CaM}\)-dependent MLCK phosphorylation of myosin light chains. In the absence of an increase in \([\text{Ca}^{2+}]_i\), force generation appears to be mediated by PKC.

Little is known about the mechanism by which force is maintained in smooth muscle with very low levels of ATP consumption, after \([\text{Ca}^{2+}]_i\) has decreased to resting levels and in the absence of appreciable levels of MLC phosphorylation. As discussed previously, a rise in \([\text{Ca}^{2+}]_i\) could result in the disinhibition of the acto-myosin domain by the association of \(\text{Ca}^{2+}.\text{CaM}\) with CaD and CaP. However, at low \([\text{Ca}^{2+}]_i\), phosphorylation of
CaD by PKC (118) and phosphorylation of CaP by PKC or CaM Kinase II (112) has been suggested to relieve their inhibition of acto-myosin interactions. Phosphorylation of CaD and CaP could, therefore, play a role in maintaining force in smooth muscle by allowing for acto-myosin cross bridge cycling at relatively low $[\text{Ca}^{2+}]_i$.

Two other models have been proposed for maintenance of force in smooth muscle. These include the thick filament-mediated latch bridge model of Dillon et al. (103) (see (119) for review) and the coordinate fibrillar domain model of Rasmussen et al. (see (120) for review) involving the thin (actin-tropomyosin) and intermediate filament (filamin-actin-desmin) domains of smooth muscle.

Relaxation of smooth muscle occurs by the removal of Ca$^{2+}$ from the intracellular space primarily by the action of the Ca$^{2+}$-ATPases of the plasma membrane and the sarcoplasmic reticulum. Activation of cyclic GMP-dependent protein kinase results in an increase in the ATPase activities of the plasma membrane and sarcoplasmic reticular Ca$^{2+}$-ATPases (121,122). Activation of cyclic AMP-dependent protein kinase results in an increase in the ATPase activity of only
the sarcoplasmic reticular Ca\textsuperscript{2+}-ATPase (123,124). Activation of these Ca\textsuperscript{2+}-ATPases, results in an increased sequestration of Ca\textsuperscript{2+} by the sarcoplasmic reticulum and an increased extrusion of Ca\textsuperscript{2+} from the intracellular space, leading to the relaxation of smooth muscle. It has been shown that activation of PKC also leads to an increase in the plasma membrane Ca\textsuperscript{2+}-ATPase activity, resulting in smooth muscle relaxation (125). Phosphorylation of MLCK by PKA and PKC in the absence of CaM has been shown to decrease its affinity for CaM ~10-fold (126-128). It has been proposed that the decrease in the affinity of MLCK for CaM upon phosphorylation by PKA could facilitate relaxation in smooth muscle (102,126). The physiological relevance of the phosphorylation of MLCK by PKC is unknown.

Currently, little is known about the temporal nature of CaM's interaction with its target proteins in smooth muscle and in other biological systems i.e., its rates of association with and dissociation from target proteins in response to a Ca\textsuperscript{2+} transient. This is because most studies performed to date have involved the determination of the Ca\textsuperscript{2+}- and CaM-dependence of
activation of its target proteins, but not the kinetics of CaM-target protein interaction.

Rapid kinetic studies allow the measurement of the association ($k_{on}$) and dissociation rate constants ($k_{off}$) of CaM-target protein complexes. This dissertation is a study of the rates of the Ca$^{2+}$-dependent binding of a fluorescently labeled wheat CaM, CaM$_{MIANS}$, to smooth muscle MLCK and CaD and the rates of CaM-target protein complex disruption following Ca$^{2+}$ chelation. These studies should further our understanding of the temporal nature of the association and dissociation of CaM with/from MLCK and CaD in response to a rise and fall in [Ca$^{2+}$]$_i$, during a contraction-relaxation cycle in smooth muscle.

J. Fluorescence Spectroscopy

Fluorescence spectroscopy is a powerful tool in the study of the structure and dynamics of protein molecules (for review see (129-131)). The sensitivity of fluorescence to its immediate environment, together with its high absolute sensitivity (only nanomolar concentrations of fluor are needed to generate an observable fluorescence emission) and the fact that fluorescence can be observed from clear or opaque
solids and liquids accounts for the widespread use of fluorescence as a biochemist’s tool.

Absorption of a photon by a molecule is a single instantaneous ($10^{-15}$ s) interaction. The ‘excited’ molecule can possess any one of a set of discrete amounts of energy called electronic energy levels, determined by the possible spatial distributions of its electrons which is further divided into vibrational and rotational energy levels. Most molecules in the ground state i.e. the lowest electronic energy level, are singlets (all electrons are paired). On absorbing radiation, one electron is excited to a higher energy state without a change of spin. Such states are excited singlet states and the excited state with the lowest energy is the first excited singlet.

A molecule excited to a higher electronic state such as $S_1^*$, can return to the ground state ($S_0$) in a number of ways (see Figure 3). Fluorescence occurs by a decay of the excited singlet state from the lowest vibrational level of $S_1^*$ to the ground state via the emission of a photon. Fluorescence lifetimes are generally on the order of $1 - 100$ ns. $S_1^*$ can also return to the ground state by a rapid ($10^{-12} - 10^{-15}$ s) non-radiative process called internal conversion.
Figure 3 Absorption and emission processes. The figure shows electronic and vibrational energy levels and transitions between them. $S_0$ represents the ground state, $S_1^*$ and $S_2^*$ represent the first and second excited singlet states, respectively. Absorption and emission are shown by solid lines, while processes leading to heat loss are shown by dashed lines.
In addition, the electron in the excited singlet state, $S_1^*$, can undergo a transition to an excited triplet state $T_1^*$ by a process called inter-system crossing in which electron spins are no longer paired. This process occurs in $\sim 10^{-8}$ s. The decay of $T_1^*$ to the ground state by the emission of a photon is called phosphorescence and occurs more slowly than fluorescence ($\sim 10^{-4} - 10$ s). $T_1^*$ can also decay to the ground state non-radiatively by internal conversion. The decay of an excited singlet state to the ground state by internal conversion or by inter-system crossing requires that the potential energy surfaces of the two states cross at an easily accessible energy.

Absorption of energy by a molecule in the ground state usually exceeds the energy required to thermally populate the zero vibrational level of the first excited singlet state ($S_1^*$). The excess energy of the excited singlet state is converted into thermal energy. Decay from vibrational levels of higher excited singlet states such as $S_2^*$, $S_3^*$, etc. proceeds first via thermal relaxation to the zero vibrational levels of $S_2^*$, $S_3^*$, etc. and then by internal conversion to the zero vibrational level of $S_1^*$. Fluorescence occurs only by the radiative decay of the 'excited' molecule.
from the zero vibrational level of the first excited singlet state to the ground state. The non-radiative mechanisms, internal conversion and thermal relaxation compete with fluorescence to deactivate the excited singlet state. Under the circumstances where such non-radiative processes predominate quenching of fluorescence occurs. In addition to these non-radiative processes, bi-molecular processes such as resonance energy transfer (coupling of transition dipoles of donor and acceptor molecules) and collisional and static quenching compete with fluorescence to deactivate the excited state. The result of these bimolecular processes is to decrease the quantum yield and energy of fluorescence.

Molecules that display easily observed fluorescence are called fluorophores. Typically such molecules possess low-lying excited states such that excitation is possible with radiation wavelengths greater than 220 nm i.e., energies less than 550 kJ mol\(^{-1}\).

There are three types of fluorophores in proteins— intrinsic, co-enzymic and extrinsic. The intrinsic fluorophores are the aromatic side chains of phenylalanine, tyrosine and tryptophan residues.
Coenzymic fluorophores are the fluorescent co-enzymes of some proteins such as reduced nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD) and pyridoxal phosphate. Extrinsic fluorophores (probes) are fluorescent molecules that are inserted into the protein or peptide of interest. Extrinsic probes have to meet three specific criteria:

(1) The probe should be non-covalently or covalently attached at a unique location on the protein.

(2) The fluorescence properties of the probe should be sensitive to the structure and dynamics of its environment.

(3) The insertion of the probe should not appreciably alter the features of the protein under investigation (e.g., its biological activity).

Many commercially available useful probes (see Figure 4) absorb light of wavelengths > 310 nm and fluoresce in the range 420-700 nm, thereby eliminating interference from the intrinsic fluorescence of aromatic amino acid residues of proteins. Some probes contain reactive groups that allow for their covalent attachment to the protein or peptide of interest at specific reactive amino acid residues such as cysteine.
(e.g., MIANS), lysine or tyrosine. The aromatic probes such as ANS, TNS, DANS (see Figure 4) are bound non-covalently to hydrophobic domains on target proteins. These probes contain rigid naphthalene ring systems that greatly increase the probability of fluorescence emission by decreasing the probability of competing, non-radiative thermal relaxation processes.

The quantum yield \( Q_F \) and energy of fluorescence of these fluorophores are largely affected by two important environmental factors, namely, solvent polarity and viscosity.

The fluorescence quantum yield, \( Q_F \), is defined as the ratio of the number of photons emitted to the number of photons absorbed. As fluorescence normally occurs from the zero vibrational level of \( S_1^* \) and the most likely absorption transition is of greater energy, fluorescence displays a Stokes shift i.e., a shift of the fluorescence emission spectrum to longer wavelengths compared with absorption. For many fluorophores, the Stokes shift is found to depend on the polarity and viscosity of the solvent. The two most significant contributions to 'polarity' are the polarizability of solvent by the excited molecule and dipole-dipole interactions between the excited molecule
Figure 4 Some commonly used fluorescent probes.
and solvent. Excitation of a molecule from the ground state to an excited state by the absorption of a photon, results in an electronic transition that alters the distribution of electrons within the molecule (i.e., its polarization). This results in an increase in the magnitude of its dipole moment and consequently, nearby solvent molecules reorient in response. The greater the polarizability of the solvent, the greater is the lowering of the excited state energy and the consequent shift of emission to lower frequency because alignment of the dipoles of the excited molecule and solvent increases the probability of deactivation of the excited state energy and a reduction of fluorescence quantum yield (Q_F). For example, ANS has an emission maximum at 515 nm in water with a Q_F of 0.04. In the presence of n-butanol, a non-polar solvent, the Q_F increases to 0.566 and the emission maximum blue-shifts to 464 nm from 515 nm i.e. energy of fluorescence emission is increased.

An increase in solvent viscosity decreases the ability of solvent dipoles to interact with the excited state and deactivate the fluorophore during the lifetime of the excited singlet state. This results in
an increase in $Q_F$ with a blue-shift in the emission maximum of fluorescence in viscous solvents.

The sensitivity of fluors such as MIANS to the polarity of their environment can be used to report conformational changes occurring in labeled proteins upon protein or ligand binding. If the binding of ligand or protein to a labeled protein e.g., MIANS-labeled wheat CaM (CaM$_{MIANS}$), results in a change in the environment of the probe, the quantum yield ($Q_F$) and energy of fluorescence are altered. Following an increase in the hydrophobicity of the probe's environment, both the $Q_F$ and the energy of emission are usually increased. The reverse occurs when the probe is exposed to a hydrophilic environment. Target protein binding to CaM$_{MIANS}$ results in an increase in its $Q_F$ and a small blue-shift in its fluorescence emission.

Extrinsic probes have also been used to measure distances between groups in a protein, to determine the extent of flexibility of a protein and to measure the rate of very rapid conformational transitions. These utilities of extrinsic probes make them a very valuable tool in fluorescence studies of biological systems.
A. Protein and Enzyme Purification

CaM was purified from wheat germ as described by Strasburg et al. (132) with the modifications of Mills et al. (133). Acetone-extracted wheat germ was homogenized in EDTA-containing buffer and clarified by centrifugation. CaM was acid-precipitated (5% TCA) and redissolved by the addition of Tris base. Solid \((\text{NH}_4)_2\text{SO}_4\) was added up to 55% saturation and the supernatant obtained upon centrifugation was dialysed to remove \((\text{NH}_4)_2\text{SO}_4\) and applied to a Phenyl Sepharose CL-4B column in the presence of \(\text{Ca}^{2+}\). Wheat CaM was eluted with an EGTA-containing buffer and further purified by ion-exchange chromatography using DEAE-spectragel.

CaM was purified from bovine brain according to the procedure of Gopalakrishna and Anderson (134). Briefly, bovine brains were homogenized in EGTA-containing buffer and clarified by centrifugation. Solid \((\text{NH}_4)_2\text{SO}_4\) was added to the homogenate up to 50%
saturation and clarified by centrifugation. Brain CaM was precipitated from the supernatant by decreasing the pH of the supernatant to CaM's isoelectric point (pH = 4.1). The pellet of brain CaM was resuspended in buffer (pH = 7.5) and purified by hydrophobic interaction chromatography using Phenyl Sepharose CL-4B followed by hydroxylapatite chromatography or ion-exchange chromatography using DEAE-Spectragel.

MLCK was purified from chicken gizzards according to the method of Adelstein and Klee (97). MLCK was extracted from homogenized gizzards in the presence of high concentrations of MgCl$_2$ (25 mM) and EGTA (5 mM) in 40 mM Tris-HCl buffer, pH = 7.5. Solid (NH$_4$)$_2$SO$_4$ was added to the crude extract of MLCK up to 40% saturation and MLCK was precipitated from the supernatant obtained upon centrifugation by the addition of (NH$_4$)$_2$SO$_4$ up to 60% saturation. The precipitate of MLCK was re-suspended in 15 mM Tris-HCl buffer, pH = 7.5, 0.5 M NaCl, 5 mM EGTA, 1 mM EDTA and applied to a Sephacryl S-300 gel filtration column. Fractions were assayed for enzyme activity using smooth muscle myosin light chains. The fractions containing peak MLCK activity were pooled and applied to a DEAE-Sephacel ion-exchange column. The kinase was eluted with a linear gradient
(0.02 - 0.6 M) of NaCl and affinity-purified using a column of brain CaM covalently attached to Affi-gel 15. The purity of MLCK was assessed by 7% SDS-polyacrylamide gel electrophoresis and its specific activity was determined by measuring the amount of [γ-32P]ATP incorporated into myosin light chains.

CaD was purified from chicken gizzards by a slight modification of the procedure of Lynch and Bretscher (135). Briefly, chicken gizzards were homogenized in 50 mM MOPS buffer, pH = 7.0 in the presence of 0.3 M KCl, 1 mM EGTA, 0.5 mM MgCl₂ and 1mM DTT and boiled at 90° C for 2 - 5 min. in a water bath. The homogenate was cooled on ice, clarified by centrifugation and (NH₄)₂SO₄ was added up to 50% saturation. The precipitate of CaD obtained upon centrifugation was resuspended in 10 mM MOPS buffer, pH = 7.0, 1mM DTT, dialysed to remove (NH₄)₂SO₄ and applied to a DEAE-Spectragel ion-exchange column. CaD was eluted with a linear gradient (0.0 - 0.25 M) of NaCl. The final purification step involved gel filtration using a column of Spectragel Ac 44. The purity of CaD was verified by 7% SDS-polyacrylamide gel electrophoresis.
B. Fluorescent labeling of wheat CaM

Difficulties with achieving stoichiometric labeling of wheat CaM with sulfhydryl selective probes have been reported (136). We have observed that purified wheat CaM sometimes runs as a monomer (17,000 Da) and a dimer (34,000 - 35,000 Da) on 12% SDS-polyacrylamide gels suggesting the presence of an intermolecular di-sulfide bond in the dimerized wheat CaM. This intermolecular di-sulfide bond could explain the decreased sulfhydryl reactivity and decreased stoichiometry of labeling of wheat CaM with sulfhydryl selective probes. To overcome this problem, purified wheat CaM (2 mg/ml) was incubated with 6 M guanidine-hydrochloride and DTT for 24 hrs. at 4°C and exhaustively dialysed against 10 mM MOPS buffer, pH = 7.0, 90 mM KCl, and 2 mM EGTA to remove the guanidine hydrochloride and DTT. This wheat CaM was incubated with a 10-fold molar excess of MIANS for 30 hrs. at 4°C, in the presence of 2 mM EGTA. The sample was then exhaustively dialyzed against 10 mM MOPS buffer, pH = 7.0, 90 mM KCl, to remove free label. The incorporation under these conditions was 0.7 - 1.0 mol MIANS/mol CaM (assuming $E_{320} = 20,000 \text{ M} \cdot \text{cm}^{-1}$). The
fluorescently-labeled wheat CaM (CaM$_{MIANS}$) ran as a single fluorescent band on 12% polyacrylamide gels.

C. Assays for MLCK activity

MLCK activity was assayed as described by Adelstein and Klee (97). Smooth muscle light chain phosphorylation by MLCK was initiated by the addition of [γ-$^{32}$P]ATP. The reaction mixture was incubated at 25°C for 1-5 min. (see Figures 5,6 for details). The assay was terminated by the addition of an aliquot of 10% TCA and 2% sodium pyrophosphate and rapidly filtered using a Millipore filtration unit. The washed filter was counted in a Beckmann LS-7500 scintillation spectrometer.

D. Phosphorylation of MLCK by c-AMP dependent protein kinase

The catalytic subunit of protein kinase A (PKA) was purified according to the procedure of Reimann and Beham (137). Phosphorylation of MLCK (250 nM) was carried out by the addition of 10 μl of PKA (2.5 mg/ml) to 1 ml of 10 mM MOPS buffer, pH = 7.0, 90 mM KCl, 10 mM MgCl$_2$, 100 μM ATP, 200 μM EGTA at 22°C for 20 min. The reaction was stopped by placing the reaction
mixture on ice and by dilution of the phosphorylated MLCK (P-MLCK) into the requisite buffer for rapid kinetic studies. The stoichiometry of MLCK phosphorylation (1.5 mol Pi/ mol kinase) was determined by phosphorylating MLCK as described above in the presence of [γ-32P]ATP (0.01 μCi/ μl).

E. Fluorescence Titrations

Titrations of CaM\textsubscript{MIA} with MLCK and CaD were carried out at 22°C in 10 mM MOPS buffer, pH = 7.0, 90 mM KCl. Ca\textsuperscript{2+} titrations were carried out in 200 mM MOPS buffer, pH = 7.0, 90 mM KCl and 2 mM EGTA buffer to prevent pH changes. Free [Ca\textsuperscript{2+}] was calculated using the following logarithmic association constants for metals and H\textsuperscript{+} to EGTA: H\textsuperscript{+} to EGTA\textsuperscript{4-}, 9.46; H\textsuperscript{+} to HEGTA\textsuperscript{3-}, 8.85; H\textsuperscript{+} to H\textsubscript{2}EGTA\textsuperscript{2-}, 2.68; H\textsuperscript{+} to H\textsubscript{3}EGTA\textsuperscript{1-}, 2.0; Ca\textsuperscript{2+} to EGTA\textsuperscript{4-}, 11.0; Ca\textsuperscript{2+} to HEGTA\textsuperscript{3-}, 5.32; Mg\textsuperscript{2+} to EGTA\textsuperscript{4-}, 5.21; and Mg\textsuperscript{2+} to HEGTA\textsuperscript{3-}, 3.37. CaM\textsubscript{MIA} was excited at 320 nm and its fluorescence monitored at 440 nm. All fluorescence measurements were carried out in a Perkin-Elmer LS5 Spectrofluorometer.
F. Kinetic Methods

Transient-state fluorescence measurements were carried out in a stopped-flow instrument (Applied Photophysics Ltd., UK, Model SF.17 MV) with a dead time of 1.6 ms at 22°C. The sample was excited at 320 nm (for CaM$_{\text{MIANS}}$) and at 335 nm (for Quin 2) using a 150 watt Xenon arc source. The fluorescence emission was observed through 10 nm band-pass interference filters (440 nm for CaM$_{\text{MIANS}}$, 510 nm for Quin 2) obtained from Oriel Corp. A 10 mm pathlength was used in all experiments. The curve fitting program (software by A.J. King, Applied Photophysics Ltd.) uses the the non-linear Levenberg-Marquardt algorithm (138).

G. Other procedures

Protein concentrations were determined by the Bradford method using γ-globulin as the standard or by U.V. absorption using the following extinction coefficients: vertebrate CaM $E_{\%277nm}^{1\%} = 1.9$ (139), CaD $E_{\%280nm}^{1\%} = 3.3$ (106), MLCK $E_{\%278nm}^{1\%} = 11.4$ (140).
CHAPTER III

KINETICS OF CALMODULIN BINDING TO ITS SMOOTH MUSCLE TARGET PROTEINS

A. INTRODUCTION

Calmodulin (CaM) is a ubiquitous Ca$^{2+}$-binding protein found in all eukaryotic cells. It binds Ca$^{2+}$ rapidly ($t_{1/2} < 2$ ms) (141) and activates a variety of intracellular enzymes (28). As cytosolic [Ca$^{2+}$] falls, CaM dissociates from these target enzymes and they inactivate. In this manner, CaM regulates numerous Ca$^{2+}$-dependent cellular processes including smooth muscle contraction, neuro-secretion and cell motility.

CaM plays a major role in the generation and maintenance of force in smooth muscle by binding its target contractile proteins, namely, myosin light chain kinase (MLCK) and caldesmon (CaD) (93,142). The rise in smooth muscle intracellular free [Ca$^{2+}$], is separated from the generation of tension by a 200-400 ms delay generally ascribed to regulatory events that precede acto-myosin cross-bridge formation and force generation (89). During this delay, CaM must associate
with MLCK to effect myosin light chain phosphorylation (93). Presently, little is known about the rate at which CaM interacts with MLCK and CaD or the rate at which CaM dissociates from these proteins as the Ca\(^{2+}\) signal is attenuated.

Ca\(^{2+}\) dissociation from CaM has been reported to occur as a mono-exponential or bi-exponential process. Studies using CaM's intrinsic tyrosine fluorescence (64,70,71), the fluorescent Ca\(^{2+}\) indicators Quin 2 (71) and BAPTA (64) and the fluorescent probe anthroylcholine (86), suggest that Ca\(^{2+}\) dissociation from CaM occurs with rates of 10.4 - 12.6 s\(^{-1}\). Martin et al. (65) and Bayley et al. (66) have reported fast (240 s\(^{-1}\), 600 s\(^{-1}\)) and slow (24 s\(^{-1}\), 10.4 s\(^{-1}\)) rates for Ca\(^{2+}\) dissociation from CaM. \(^1\)H and \(^{43}\)Ca NMR studies have provided evidence for fast (600-1000 s\(^{-1}\)) and slow (30-40 s\(^{-1}\)) Ca\(^{2+}\) exchange sites on CaM (36,68,69). Kinetic studies of the rates of Ca\(^{2+}\) dissociation from CaM-drug / peptide complexes (65,71) have shown that high-affinity drug / peptide binding to CaM increases its affinity for Ca\(^{2+}\). Equilibrium Ca\(^{2+}\)-binding data have shown that CaM's affinity for Ca\(^{2+}\) is increased 7 to 35-fold upon complex formation with high
affinity target proteins (84-86) and the CaM-antagonist peptide melittin (83).

Plant CaM, unlike vertebrate CaM, contains a single cysteine residue at position 26 (29) or position 27 (30). We have previously labeled spinach CaM with a fluorescent, sulfhydryl selective probe MIANS (133). This labeled CaM (CaM_MIANS) is biologically active and undergoes a fluorescence increase exclusively upon target protein binding. In contrast, Dans-CaM's fluorescence is affected by Ca$^{2+}$-, drug-, peptide- and protein-binding (143-145). In this study, we use fluorescent stopped-flow techniques to determine the association and dissociation rate constants for the interaction of CaM_MIANS with MLCK, PKA-phosphorylated MLCK (P-MLCK) and CaD. We examine the rate of CaM-target protein complex disruption induced by the calcium chelator, EGTA, and the effect of MLCK and CaD on Ca$^{2+}$ dissociation rates from CaM. These studies should further our understanding of the temporal nature of the interaction between CaM and its target smooth muscle contractile proteins during an increase in intracellular [Ca$^{2+}$], which leads to the development and maintenance of force in smooth muscle.
B. Results

Brain CaM, wheat CaM and CaM\textsubscript{MIANS} activation of MLCK

Brain CaM, wheat CaM and CaM\textsubscript{MIANS} activate MLCK (100 nM) with a similar dose-dependence and maximal MLCK activity is observed near stoichiometric concentrations (100 nM) of each CaM (Figure 5). CaM\textsubscript{MIANS} stimulates MLCK's activity to ~70% of that observed with brain CaM and unlabeled wheat CaM. Brain CaM, wheat CaM and CaM\textsubscript{MIANS} all activate MLCK half-maximally at pCa = 6.55, in a cooperative manner (Hill coefficient = 4.0) (Figure 6). At pCa = 4.0, CaM\textsubscript{MIANS} stimulates MLCK activity to ~70% of that observed with brain or unlabeled wheat CaM. Thus, all three CaM's produce a similar Ca\textsuperscript{2+}-dependent activation of MLCK although the labeled CaM exhibits a slightly lower stimulation of MLCK activity.

Interaction of MLCK, P-MLCK and CaD with CaM\textsubscript{MIANS}

The increase in the fluorescence intensity of CaM\textsubscript{MIANS} (50 nM) is shown as a function of increasing concentrations of MLCK, P-MLCK and CaD in Figure 7. MLCK binds to CaM\textsubscript{MIANS} stoichiometrically with an ED\textsubscript{50} of 14 nM (F/Fo = 3.2). Titrations of lower concentrations of CaM\textsubscript{MIANS} (10 nM) with MLCK were also stoichiometric suggesting a high affinity (K\textsubscript{d} < 1 nM).
Figure 5  Calmodulin-dependence of myosin light chain kinase activation. The activation of MLCK by brain CaM (○), wheat CaM (■) and CaM_HANS (●) is shown. Phosphorylation of myosin light chains by MLCK was carried out at 25°C for 5 min. in 200 μl of 40 mM MOPS, pH = 7.2, 2 mM EGTA, 10 mM MgCl₂, 2.4 mM CaCl₂, 0.1M NaCl, 1.8 μM MLC, 100 nM MLCK, 5 μM [γ-³²P] ATP (0.5 μCi/assay) and the indicated concentrations of CaM. Reactions were terminated by the addition of 1 ml of ice cold 10% TCA, 2% sodium pyrophosphate. Each point is the mean of 2 determinations.
Figure 6  Calcium-dependence of myosin light chain kinase activation. The calcium-dependence of activation of MLCK by brain CaM (○), wheat CaM (■) and Ca$_{MIANS}$ (●) is shown. Myosin light chain phosphorylation assays were conducted at 25°C for 90 s, in 100 µl of 200 mM MOPS, pH = 7.0, 90 mM KCl, 2mM EGTA, 10 mM MgCl$_2$, 8.6 µM MLC, 6.73 nM MLCK, 100 µM [γ$^{32}$P] ATP (3.5 µCi/assay), 1 x 10$^{-7}$ M CaM with CaCl$_2$ added to give the indicated pCa. Reactions were terminated by the addition of 1 ml of ice cold 10% TCA, 2% sodium pyrophosphate. Each point is the mean of 3 determinations.
Figure 7. Interaction of smooth muscle target proteins with fluorescently-labeled wheat calmodulin. The fractional (%) increase in fluorescence of CaM_{MIIANS} (50 nM) is shown as a function of total added MLCK (■), P-MLCK (▲) and CaD (●). Titrations were conducted in 1 ml of 10 mM MOPS, pH = 7.0, 90 mM KCl, 100 μM CaCl₂. MLCK was phosphorylated (1.5 mol/mol) as described in Methods except that 10 μl of PKA (2.5 mg/ml) were added to 75 μl of MLCK (9.6 μM) and incubated at room temperature for 1 hour. 100% fluorescence increase corresponds to a 3.2-fold (+MLCK), 2.6-fold (+P-MLCK) and 1.8-fold (+CaD) enhancement, respectively. Each curve is the mean of 3 titrations.
of MLCK for CaM\textsubscript{MIANS}. P-MLCK shows a reduced affinity for CaM\textsubscript{MIANS} with an ED\textsubscript{50} of 27 nM (F/Fo = 2.6). CaD binds to CaM\textsubscript{MIANS} with much lower affinity than MLCK and P-MLCK with an ED\textsubscript{50} of 60 nM (F/Fo = 1.8). None of these target proteins increase the fluorescence of CaM\textsubscript{MIANS} in the absence of Ca\textsuperscript{2+} (+ 2 mM EGTA). Ca\textsuperscript{2+}-binding to CaM\textsubscript{MIANS} produces a small decrease (F/Fo = 0.8) in its fluorescence intensity. These results are consistent with Ca\textsuperscript{2+}-dependent target protein binding to CaM\textsubscript{MIANS}, with the order of affinity being MLCK > P-MLCK > CaD.

**Kinetics of MLCK binding to CaM\textsubscript{MIANS}**

The second order rate constant for the association of MLCK (100 nM) with increasing [CaM\textsubscript{MIANS}] was determined by approximating pseudo-first order reaction conditions. A plot of k\textsubscript{obs} vs. [CaM\textsubscript{MIANS}] shows that the rate of complex formation increases with increasing [CaM\textsubscript{MIANS}], yielding a second order rate constant (k\textsubscript{on}) of 2.8 x 10\textsuperscript{7} ± 0.18 x 10\textsuperscript{7} M\textsuperscript{-1}s\textsuperscript{-1} (Figure 8, inset). The rates of complex formation were identical whether MLCK +Ca\textsuperscript{2+} was rapidly mixed with CaM\textsubscript{MIANS} + Ca\textsuperscript{2+} or MLCK + CaM\textsubscript{MIANS} + EGTA was rapidly mixed with Ca\textsuperscript{2+}. Thus, the association of MLCK with CaM\textsubscript{MIANS} is not
Figure 8  Kinetics of myosin light chain kinase binding to CaM.  CaM_{MIANS} (200 nM) and MLCK (250 nM) or P-MLCK (250 nM) in 10 mM MOPS buffer, pH = 7.0, 90 mM KCl, 0.2 mM EGTA, 0.4 mM CaCl$_2$, 10 mM MgCl$_2$, 100 $\mu$M ATP was rapidly mixed with a 20-fold excess of unlabeled CaM (4 $\mu$M) in the same buffer and the corresponding fluorescence decrease was monitored as a function of time.  MLCK was phosphorylated (1.5 mol/mol) by PKA as described in Methods.  Control experiments (CaM_{MIANS} + MLCK or CaM_{MIANS} + P-MLCK vs. Ca$^{2+}$) were performed and the data shown are the mean of 6-8 traces with averaged, control traces subtracted in each case (variance < 1 X 10$^{-5}$).  The inset shows the plot of the observed rate ($k_{obs}$) of CaM_{MIANS} - MLCK complex formation vs. [CaM_{MIANS}] ($r = 0.99$).  The $k_{obs}$ for each concentration of CaM_{MIANS} was determined from the rate of fluorescence increase that occurs when MLCK (100 nM) is rapidly mixed with each concentration of CaM_{MIANS} (300 - 1100 nM) in the above buffer.  Control experiments (CaM_{MIANS} + Ca$^{2+}$ vs. Ca$^{2+}$) indicated that we were observing ~90% of the fluorescence increase at the highest [CaM_{MIANS}] used.  The data (n = 6-8 traces) were fit with mono-exponential curves (variance < 1 X 10$^{-5}$).
Figure 8
rate-limited by the binding of Ca\textsuperscript{2+} to CaM (data not shown).

The dissociation rate constant (k\textsubscript{off}) of the Ca\textsuperscript{M}\textsubscript{MIANS-MLCK} complex was determined by displacing MLCK from the complex with a 20-fold excess of brain CaM over [Ca\textsuperscript{M}\textsubscript{MIANS}] (Figure 8). Complex dissociation occurs very slowly, with a rate of 0.0045 ± 0.00003 s\textsuperscript{-1}. The K\textsubscript{d} of MLCK for Ca\textsuperscript{M}\textsubscript{MIANS} determined from these on- and off-rates (K\textsubscript{d} = k\textsubscript{off}/k\textsubscript{on}) is 0.16 nM. The high affinity CaM-antagonist peptide, melittin, (K\textsubscript{d} = 3 nM) was also used to disrupt the Ca\textsuperscript{M}\textsubscript{MIANS-MLCK} complex. Complex disruption occurred slowly, with a rate of 0.006 ± 0.0002 s\textsuperscript{-1}, when melittin was present in 5 to 15-fold excess (1 - 3 \mu M) over Ca\textsuperscript{M}\textsubscript{MIANS} (data not shown).

**Kinetics of P-MLCK binding to Ca\textsuperscript{M}\textsubscript{MIANS}**

To determine the effect of PKA-phosphorylation of MLCK on its rates of association with and dissociation from Ca\textsuperscript{M}\textsubscript{MIANS}, MLCK was phosphorylated to 1.5 mol Pi/mol MLCK. Figure (8) compares the rates of MLCK and P-MLCK dissociation from Ca\textsuperscript{M}\textsubscript{MIANS} with a 20-fold excess of brain CaM over [Ca\textsuperscript{M}\textsubscript{MIANS}]. P-MLCK dissociates from Ca\textsuperscript{M}\textsubscript{MIANS} nine times more rapidly than MLCK (0.04 ± 0.0001 s\textsuperscript{-1} compared to 0.0045 ± 0.00003 s\textsuperscript{-1}) consistent
with a reduced affinity of MLCK for CaM_{MIANS}, upon phosphorylation of MLCK. The second order rate constant for the association of P-MLCK with CaM_{MIANS} (using CaM_{MIANS} in 9-fold molar excess over P-MLCK [125 nM]) was determined to be $8.0 \times 10^6 \pm 0.2 \times 10^6 \text{M}^{-1}\text{s}^{-1}$ (data not shown). Thus, PKA phosphorylation of MLCK results in an ~3.5-fold decrease in its rate of association with CaM_{MIANS} and a 9-fold increase in its rate of dissociation from CaM_{MIANS}. This results in a 32-fold decrease in MLCK’s affinity for CaM_{MIANS} ($K_d = 5.0 \text{ nM}$) upon phosphorylation.

**Kinetics of CaD binding to CaM_{MIANS}**

The second order rate constant for the association of CaD [400 nM] with increasing [CaM_{MIANS}] was determined by approximating pseudo-first order reaction conditions. A plot of $k_{obs}$ vs. [CaM_{MIANS}] shows that the rate of complex formation ($k_{on}$) increases with increasing [CaM_{MIANS}], yielding a second order rate constant of $5.3 \times 10^8 \pm 0.33 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ (Figure 9, inset). The rates of complex formation were identical whether CaD + Ca$^{2+}$ was rapidly mixed with CaM_{MIANS} + Ca$^{2+}$ or CaD + CaM_{MIANS} + EGTA was rapidly mixed with Ca$^{2+}$. Thus, the rate of association of CaD with
Figure 9 Kinetics of caldesmon binding to calmodulin. CaM_MIANS (200 nM) and CaD (1 μM) in 10 mM MOPS buffer, pH = 7.0, 90 mM KCl, 500 μM CaCl₂ was rapidly mixed with CaM (4 μM) in the same buffer. Control experiments (CaM_MIANS + CaD vs. Ca²⁺) were performed and the data shown is the mean of 8 traces with averaged, control traces subtracted from it (variance < 1 x 10⁻⁵). The inset shows the plot of the observed rate (k_obs) of CaM_MIANS-CaD complex formation vs. [CaM_MIANS] (r = 0.99). The k_obs for each concentration of CaM_MIANS was determined from the rate of the fluorescence increase that occurs when CaD (0.4 μM) in 10 mM MOPS (0.4 μM) buffer pH = 7.0, 90 mM KCl, 200 μM CaCl₂ is rapidly mixed with each concentration of CaM_MIANS (0.4 - 2.8 μM) in the same buffer. Control experiments (CaM_MIANS + Ca²⁺ vs. Ca²⁺) indicated that when the [CaM_MIANS] was increased 5 to 7-fold over [CaD], ~75% of the total amplitude change was lost in the dead time of the instrument. The data (n = 6-8 traces) were fit with mono-exponential curves (variance < 1 X 10⁻⁵).
CaM_MIANS is not rate-limited by the binding of Ca^{2+} to CaM (data not shown).

The dissociation rate constant (k_{off}) of the CaM_MIANS-CaD complex was determined by displacing CaD from the complex with a 20-fold molar excess of brain CaM over [CaM_MIANS] (Figure 9). Complex dissociation occurred very rapidly with a rate of 61 ± 0.9 s\(^{-1}\). The K_d of CaD for CaM_MIANS determined from these on- and off-rates is 115 nM. Thus, CaM_MIANS dissociates more rapidly (61 s\(^{-1}\)) from the lower affinity CaD than from the higher affinity MLCK (0.004 s\(^{-1}\)) or P-MLCK (0.04 s\(^{-1}\)). Melittin (0.25 - 1 \mu M) disruption of the CaM_MIANS-CaD complex also occurred with a similar fast rate of 40 ± 0.2 s\(^{-1}\) (data not shown).

**EGTA disruption of CaM_MIANS-target protein complexes**

MLCK, P-MLCK and CaD have very different affinities for CaM (0.16 nM, 5.0 nM and 115 nM, respectively). We wished to determine if these differences in affinity for CaM would affect the rate at which EGTA disrupts each CaM-target protein complex. EGTA-disrupts the CaM_MIANS-MLCK complex at 3.5 ± 0.03 s\(^{-1}\), the CaM_MIANS-P-MLCK complex at 6.5 ± 0.05 s\(^{-1}\) and the CaM_MIANS-CaD complex at 13.5 ± 0.15 s\(^{-1}\) (Figure 10). Thus, for these target proteins, EGTA disruption
Figure 10  Kinetics of calmodulin-target protein complex disruption by a calcium chelator. CaM-MIANS (200 nM) and MLCK (250 nM) or P-MLCK (250 nM) or CaD (1.0 μM) in 10 mM MOPS buffer, pH = 7.0, 90 mM KCl, 100 μM CaCl₂, 0.25 mM MgCl₂, 2.5 μM ATP was rapidly mixed with 5 mM EGTA in the same buffer (−100 μM CaCl₂). Control experiments (CaM-MIANS + target protein +Ca²⁺ vs. Ca²⁺) indicated that we were able to monitor 100% of the EGTA-induced fluorescence change. The data (n = 6-8 traces) were fit with mono-exponential curves (variance < 1 X 10⁻⁵).
of higher affinity CaMMIANS-target protein complexes occurs more slowly than the disruption of lower affinity CaMMIANS-target protein complexes.

**Calcium dissociation from CaM and CaM-target protein complexes**

When Quin 2 is rapidly mixed with a Ca$^{2+}$-calcium binding protein complex, its fluorescence increases at a rate which is equal to the rate of Ca$^{2+}$ dissociation from that Ca$^{2+}$ binding protein (146). Free (unbound) Ca$^{2+}$ binds to Quin 2 very rapidly ($k_{on} = 7.5 \times 10^8$ M$^{-1}$s$^{-1}$) (66) and is not rate-limiting. In order to determine the effect of target protein binding on CaM’s affinity for Ca$^{2+}$, we monitored Ca$^{2+}$ dissociation from wheat CaM in the absence and presence of target proteins, using the fluorescent Ca$^{2+}$ chelator Quin 2. Ca$^{2+}$ (10 μM) dissociates from wheat CaM (0.5 μM) as a single exponential process with a rate of $30 \pm 0.7$ s$^{-1}$ (Figure 11). MLCK produced a dramatic decrease in the rate of Ca$^{2+}$ dissociation from CaM ($6 \pm 0.1$ s$^{-1}$) while CaD had little effect on the rate of Ca$^{2+}$ dissociation from CaM ($28 \pm 0.4$ s$^{-1}$) (Figure 11). Neither CaD nor MLCK produced any observable change in Quin 2 fluorescence in the absence of CaM. The amplitude of the Quin 2 - Ca$^{2+}$ signal observed in the presence of
Figure 11 Kinetics of calcium dissociation from calmodulin and calmodulin-target protein complexes. Wheat CaM (0.5 µM), wheat CaM + MLCK (0.6 µM) or wheat CaM + CaD (1.5 µM) in 10 mM MOPS buffer, pH = 7.0, 90 mM KCl, 10 µM CaCl₂, 0.1 mM DTT was rapidly mixed with Quin 2 (80 µM) in the same buffer (-10 µM CaCl₂). Control traces (target protein + Ca²⁺ or Ca²⁺ vs. Quin 2) were subtracted from the data (n = 6-8 traces) and the data were fit with mono-exponential curves (variance < 1 X 10⁻⁵).
MLCK or CaD was double that observed with wheat CaM alone. However, only the higher affinity target protein MLCK produced a dramatic 5-fold decrease (30 s\(^{-1}\) to 6 s\(^{-1}\)) in the rate of Ca\(^{2+}\) dissociation from CaM.

C. DISCUSSION

CaM isolated from many organisms shows a highly conserved primary structure. Brain and wheat CaM's are 90 % homologous (133/148 amino acid residues), with numerous conservative substitutions (30,32). Plant CaM and brain CaM exhibit identical activation of cyclic nucleotide phosphodiesterase (133,147), erythrocyte Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase (132,147) and calcineurin (133). Our studies show that brain CaM, wheat CaM and CaM\(_{\text{MIANS}}\) activate MLCK with a similar Ca\(^{2+}\)-dependence (half-maximal at pCa = 6.55, Hill coefficient = 4.0) and dose-dependence, with maximal activation occurring near stoichiometric concentrations of each CaM. Labeling of wheat CaM with MIANS causes a 30% decrease in its ability to maximally activate MLCK compared with unlabeled wheat and brain CaM's. However, this 30% decrease in the ability of CaM\(_{\text{MIANS}}\) to maximally stimulate MLCK activity, has no bearing upon our
kinetic studies which characterize the binding of MLCK to CaM\textsubscript{MIANS} and not its activation by CaM\textsubscript{MIANS}. Zot et al. (136) have shown that labeling of wheat CaM with the fluorescent probe IAANS decreases its ability to maximally activate skeletal muscle MLCK by ~32% and Kosk-Kosicka et al. (148) have shown that labeling of spinach CaM with MIANS decreases its ability to activate erythrocyte Ca\textsuperscript{2+}-Mg\textsuperscript{2+}-ATPase by ~20%. The small decrease (~30%) in the ability of MIANS-labeled wheat CaM to maximally activate smooth muscle MLCK may result from the fluorescent label at Cys 27 interfering with an interfacial site of the CaM-MLCK complex.

MLCK, P-MLCK and CaD bind CaM\textsubscript{MIANS} in the presence of Ca\textsuperscript{2+} producing large increases in its fluorescence intensity. A comparison of the ED\textsubscript{50}'s of these target proteins binding to CaM\textsubscript{MIANS} suggests that the rank order of affinity for CaM is MLCK > P-MLCK > CaD.

MLCK associates with CaM\textsubscript{MIANS} with a second order rate constant (k\textsubscript{on}) of 2.8 x 10\textsuperscript{7} M\textsuperscript{-1}s\textsuperscript{-1} and dissociates with a rate of 0.0045 s\textsuperscript{-1}. While the association and dissociation rates of this complex have not been previously reported, Chau et al. (64) have reported a similar rate of 0.003 s\textsuperscript{-1} for the dissociation of CaM from the high affinity (K\textsubscript{d} = 0.1 nM) CaM-PDE complex.
The dissociation rate constant ($k_{\text{off}}$) of 0.0045 s$^{-1}$ for the CaM$_{\text{MIANS}}$-MLCK complex is consistent with a high affinity of MLCK for CaM; $K_d = 0.16$ nM ($K_d = k_{\text{off}}/k_{\text{on}}$). This $K_d$ value of MLCK for CaM$_{\text{MIANS}}$ is in reasonable agreement with previously reported $K_d$'s of 0.5 - 1 nM for the CaM-smooth muscle MLCK interaction (102,150,151). The high affinity CaM-antagonist peptide, melittin, ($K_d = 3$ nM) (82) has often been used as a model for CaM-target protein interactions. In the presence of Ca$^{2+}$, melittin binds to CaM$_{\text{MIANS}}$ without increasing its fluorescence and prevents MLCK and CaD from binding and increasing the fluorescence of CaM$_{\text{MIANS}}$. Melittin (1 - 3 μM) disrupted the CaM$_{\text{MIANS}}$-MLCK complex at a rate of 0.006 ± 0.0002 s$^{-1}$ (data not shown). Thus, both melittin and excess unlabeled CaM disrupt the CaM$_{\text{MIANS}}$-MLCK complex with similar rates (see Table II).

PKA-phosphorylation of MLCK results in a shift in the ED$_{50}$ of its fluorescence enhancement of CaM$_{\text{MIANS}}$ from 14 nM to 27 nM, suggesting a decrease in the affinity of P-MLCK for CaM$_{\text{MIANS}}$. Consistent with this, phosphorylation of MLCK by PKA substantially alters the kinetics of its interaction with CaM$_{\text{MIANS}}$ producing a 3.5-fold decrease in its rate of association with
Table II  Summary of the kinetics of calmodulin binding to its target proteins in smooth muscle.

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>$K_{on}$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_{off}$ (CaM) (s$^{-1}$)</th>
<th>$K_{off}$ (melittin) (s$^{-1}$)</th>
<th>$K_d = K_{off}/K_{on}$ (nM)</th>
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</thead>
<tbody>
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<td>MLCK</td>
<td>$2.8 \times 10^7$</td>
<td>0.0045</td>
<td>0.006</td>
<td>0.16</td>
</tr>
<tr>
<td>P-MLCK</td>
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<td>0.04</td>
<td>n.d.</td>
<td>5.0</td>
</tr>
<tr>
<td>Caldesmon</td>
<td>$5.3 \times 10^8$</td>
<td>61</td>
<td>40</td>
<td>115</td>
</tr>
</tbody>
</table>
CaM_{MIANS} \ (k_{on} = 8 \times 10^6 \text{ M}^{-1}\text{s}^{-1}) \text{ and an } \sim 9\text{-fold increase in its rate of dissociation } (k_{off} = 0.04 \text{ s}^{-1}) \text{ from CaM}_{MIANS}. \text{ This results in a shift in the } K_d \text{ of MLCK for CaM}_{MIANS} \text{ from } 0.16 \text{ nM to 5 nM upon phosphorylation. Our results are consistent with Kamm and Stull's (102) and Conti and Adelstein's (150) reports of a shift in the } K_d \text{ of MLCK for CaM from 1 nM to 10 or 20 nM upon PKA-phosphorylation of MLCK. Therefore, phosphorylation of MLCK decreases its affinity for CaM by producing a 9-fold increase in its dissociation rate from CaM and a 3.5-fold decrease in its association rate with CaM (see Table II).}

CaD associates with CaM_{MIANS} \text{ with a second order rate constant } (k_{on}) \text{ of } 5.3 \times 10^8 \text{ M}^{-1}\text{s}^{-1} \text{ and dissociates with a rate of } 61 \text{ s}^{-1}. \text{ The } K_d \text{ of CaD for CaM}_{MIANS} \text{ determined from these studies is } 115 \text{ nM which is in reasonable agreement with the } K_d \text{ of 75 nM reported by Malencik et al. (152). Melittin (0.25 - 1 \mu M) disrupts the CaM}_{MIANS}-CaD \text{ complex with a rate of } 40 \pm 0.2 \text{ s}^{-1} \text{ (data not shown). Thus, both melittin and excess, unlabeled CaM disrupt this complex with approximately similar rates (see Table II).}

EGTA disrupts the CaM_{MIANS} - target protein complexes producing a complete reversal of the
**Table III** Summary of the rates of calcium chelator-induced disruption of calmodulin-target protein complexes.

<table>
<thead>
<tr>
<th>Target Protein</th>
<th>Complex dissociation by EGTA (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>MLCK</td>
<td>3.5</td>
</tr>
<tr>
<td>P-MLCK</td>
<td>6.5</td>
</tr>
<tr>
<td>CaD</td>
<td>13.5</td>
</tr>
</tbody>
</table>
fluorescence increases that occur upon their binding CaM•MIANS. EGTA-disruption of the CaM•MIANS-MLCK, CaM•MIANS-P-MLCK and CaM•MIANS-CaD complexes occur with rates of 3.5 s⁻¹, 6.5 s⁻¹ and 13.5 s⁻¹, respectively (see Table III). Our rate for the EGTA-disruption of the CaM•MIANS-MLCK complex (3.5 s⁻¹) agrees with the rate obtained by Johnson et al. (46) for EGTA-disruption of the CaM-skeletal muscle MLCK complex (2 s⁻¹) and with the rate of skeletal muscle MLCK inactivation (1 s⁻¹), upon Ca²⁺ chelation, reported by Stull et al. (96).

The effect of target protein binding on CaM's Ca²⁺ affinity, was determined using Quin 2 fluorescence. In the absence of target protein and at 10 - 40 µM added Ca²⁺, calcium dissociates from wheat CaM (0.5 µM) as a single exponential process with a rate of 30 s⁻¹. Martin et al. (65) and Bayley et al. (66) have used Quin 2 and ANS fluorescence and measured fast (240 - 600 s⁻¹) and slow (10 - 24 s⁻¹) Ca²⁺ dissociation rates from the lower affinity, N-terminal and the higher affinity, C-terminal sites of brain CaM, respectively. We saw no evidence for the fast Ca²⁺ dissociation rates (240-600 s⁻¹) that they reported. It is possible that the rate of Ca²⁺ dissociation from the low affinity
Figure 12  Kinetics of the EGTA-induced decrease in the intrinsic tyrosine fluorescence of wheat CaM. Wheat CaM (4.0 μM) in 10 mM MOPS buffer, pH = 7.0, 90 mM KCl, 100 μM CaCl₂, was rapidly mixed with 5 mM EGTA in the same buffer (-100 μM CaCl₂). Control experiments (wheat CaM + Ca²⁺ vs. Ca²⁺) indicated that we were monitoring 100% of the EGTA-induced, 1.8-fold tyrosine fluorescence decrease. The data (n = 6 traces) was fit with a mono-exponential curve (variance < 1 X 10⁻⁵).
sites of CaM is too fast to detect with our stopped-flow instrument.

The rate of Ca\(^{2+}\) dissociation from the C-terminal, high affinity sites of brain CaM has been measured using the intrinsic tyrosine fluorescence of CaM (64,70,71). Wheat CaM has a single C-terminal tyrosine (Tyr 138) (30). EGTA induces a 1.8-fold decrease in wheat CaM's tyrosine fluorescence with a rate of 34 ± 0.31 s\(^{-1}\) (Figure 12). This value is in good agreement with the rate of Ca\(^{2+}\) dissociation from wheat-CaM measured using Quin 2 fluorescence (30 s\(^{-1}\)) and suggests that Quin 2 is reporting Ca\(^{2+}\) dissociation from the high affinity Ca\(^{2+}\) binding sites. Our rate for the mono-exponential dissociation of Ca\(^{2+}\) from wheat CaM (30 s\(^{-1}\)) is similar to previously reported rates of 10 s\(^{-1}\) - 12.8 s\(^{-1}\) determined using vertebrate CaM’s tyrosine fluorescence (64,70,71), dansylated CaM (70), the fluorescent anthroylcholine (86) and the fluorescent Ca\(^{2+}\) indicators BAPTA (64) and Quin 2 (71). We find that the EGTA-induced decrease in brain CaM’s tyrosine fluorescence occurs at a rate of 9.4 ± 0.2 s\(^{-1}\) and that the Ca\(^{2+}\)-dissociation rate from brain CaM measured using Quin 2 fluorescence occurs at a rate of 8.03 ± 0.16 s\(^{-1}\) (data not shown). These results are in
good agreement with the studies with brain CaM reported above. Our results are also consistent with the monoexponential, Ca\(^{2+}\)-dissociation rates from brain CaM obtained by Suko et al. (71) using Quin 2 fluorescence (13.4 s\(^{-1}\)) and CaM's intrinsic tyrosine fluorescence (12.8 s\(^{-1}\)).

The higher affinity MLCK, produced a 5-fold decrease in the rate of Ca\(^{2+}\) dissociation from CaM (30 s\(^{-1}\) to 6 s\(^{-1}\)) while the lower affinity CaD, produced only a slight decrease in the rate of Ca\(^{2+}\) dissociation from CaM (30 s\(^{-1}\) to 28 s\(^{-1}\)) (see Table IV). The amplitude of the Quin 2 fluorescence signal seen upon Ca\(^{2+}\) chelation from the CaM-MLCK and the CaM-CaD complexes was ~2-fold greater than the Quin 2 fluorescence signal observed upon chelation of Ca\(^{2+}\) from wheat CaM alone. This suggests that target protein binding to CaM in the presence of 10 μM added Ca\(^{2+}\), increases the degree of saturation of CaM's Ca\(^{2+}\) sites by increasing its Ca\(^{2+}\) affinity. Alternatively, the rate of Ca\(^{2+}\) dissociation from the low affinity N-terminal Ca\(^{2+}\) binding sites could be sufficiently reduced by target protein so that dissociation of Ca\(^{2+}\) from these sites now contributes to the amplitude of the observed Quin 2 fluorescence signal.
Table IV  Summary of the rates of calcium dissociation from calmodulin-target protein and calmodulin-peptide complexes.

<table>
<thead>
<tr>
<th>CaM-target protein/peptide complex</th>
<th>Rate of Ca$^{2+}$ dissociation ($s^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaM-MLCK</td>
<td>6</td>
</tr>
<tr>
<td>CaM-CaD</td>
<td>28</td>
</tr>
<tr>
<td>CaM-melittin</td>
<td>5</td>
</tr>
</tbody>
</table>
Melittin mimicked the effect of MLCK on the rate of Ca$^{2+}$-dissociation from CaM, producing a 6-fold decrease (30 s$^{-1}$ to 5 s$^{-1}$) in its dissociation rate and a doubling of the amplitude of the Quin 2 fluorescence signal obtained in the absence of melittin (data not shown). Thus, high affinity target protein- and peptide-binding to CaM produce dramatic (5 to 6-fold) decreases in the rates of dissociation of Ca$^{2+}$ from CaM. Chau et al. (64) have reported a rate of 3.5 - 4.6 s$^{-1}$ for the initial rapid Ca$^{2+}$ release from the high affinity CaM-PDE complex using the fluorescent Ca$^{2+}$ chelator BAPTA. Schimmerlik et al. (86) have reported that TnI binding to vertebrate CaM decreases the rate of dissociation of Ca$^{2+}$ from CaM from 10 s$^{-1}$ to 6 s$^{-1}$, using the fluorescent probe anthroylcholine. Martin et al. (65) have shown that TFP produces a 6.5-fold decrease (7.1 s$^{-1}$ to 1.08 s$^{-1}$) in the rate of dissociation of Ca$^{2+}$ from CaM. Our data showing an increase in CaM's Ca$^{2+}$ affinity upon high affinity target protein binding are in agreement with equilibrium Ca$^{2+}$-binding data which suggest a 7 to 35-fold increase in CaM's Ca$^{2+}$ affinity upon the binding of melittin (82), troponin I (83), cyclic nucleotide phosphodiesterase (84) and skeletal muscle MLCK (85).
Consistent with high affinity target protein binding increasing CaM's Ca\(^{2+}\) affinity, we found that EGTA-induced disruption of the CaM\(_{M\text{IANS}}\)-MLCK and CaM\(_{M\text{IANS}}\)-P-MLCK complexes occurs more slowly than that of the CaM\(_{M\text{IANS}}\)-CaD complex (3.5 s\(^{-1}\), 6.5 s\(^{-1}\) vs. 13.5 s\(^{-1}\), respectively). This suggests that the binding of the higher affinity MLCK (K\(_d\) = 0.16 nM) and P-MLCK (K\(_d\) = 5.0 nM) to CaM\(_{M\text{IANS}}\) results in a greater increase in CaM's Ca\(^{2+}\) affinity than the binding of the lower affinity CaD (K\(_d\) = 115 nM). A comparison of the rates of Ca\(^{2+}\) dissociation from the CaM-MLCK (6 s\(^{-1}\)) and CaM-CaD (28 s\(^{-1}\)) complexes with the rates of EGTA-induced disruption of the CaM\(_{M\text{IANS}}\)-MLCK complex (3.5 s\(^{-1}\)) and the CaM\(_{M\text{IANS}}\)-CaD complex (13.5 s\(^{-1}\)) shows that Ca\(^{2+}\) dissociation from each CaM-target protein complex occurs ~2-fold faster than the rate of EGTA-induced complex disruption. It is likely that some rate-limiting structural change must occur after Ca\(^{2+}\) dissociation from the CaM-target protein complex to allow complex disruption.

In conclusion, the association rate constants for the CaM\(_{M\text{IANS}}\)-MLCK and CaM\(_{M\text{IANS}}\)-CaD complexes determined from our studies suggest that following a transient rise in smooth muscle intracellular [Ca\(^{2+}\)], CaM could
bind both MLCK and CaD within the -200-400 ms interval that precedes the generation of force (89). In addition, the higher affinity CaM target proteins produce larger increases in CaM's Ca$^{2+}$ affinity and decrease the rates at which the complexes disrupt upon Ca$^{2+}$ chelation. The rates of EGTA-disruption of the CaM$_{MIANS}$-MLCK ($t_{1/2} = 0.2$ s), CaM$_{MIANS}$-P-MLCK ($t_{1/2} = 0.1$ s) and CaM$_{MIANS}$-CaD ($t_{1/2} = 0.05$ s) complexes are, however, quite rapid. A decrease in intracellular [Ca$^{2+}$] in smooth muscle would result in the dissociation of CaM from MLCK, P-MLCK and CaD, with rates that are sufficiently rapid to account for their participation in the process of smooth muscle relaxation ($t_{1/2} \approx 24$ s) (93). These kinetic studies should help to further our understanding of the temporal nature of the Ca$^{2+}$-dependent events that modulate the contraction-relaxation cycle of smooth muscle during a Ca$^{2+}$ transient.
LIST OF REFERENCES


