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CHARACTERIZING THE RESPONSE OF CALCIUM SIGNAL TRANSUDERS TO GENERATED CALCIUM TRANSIENTS AND MODIFYING CA^{2+} AND MG^{2+} AFFINITY USING SITE-DIRECTED MUTAGENESIS OF EF-HANDS

DISSERTATION

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

By

Jonathan P. Davis

The Ohio State University

1999

Dissertation Committee

Professor J. David Johnson, Advisor

Professor Ruth Altschuld

Professor Jack Rall

Professor Peter Reiser

Approved by

Adviser

Biophysics Graduate Program
ABSTRACT

Cellular Ca$^{2+}$ transients and Ca$^{2+}$-binding proteins regulate physiological phenomena as diverse as muscle contraction, neurosecretion and cell division. When Ca$^{2+}$ chelators with slow Ca$^{2+}$ on-rates (EGTA and Mg$^{2+}$-EDTA) are rapidly mixed with Ca$^{2+}$, the [Ca$^{2+}$] initially rises and then falls as these chelators bind Ca$^{2+}$. Using this approach, Ca$^{2+}$ transients of various amplitudes and durations were generated in a stopped-flow-apparatus to determine the response of Ca$^{2+}$ indicators (fluo-3 and MF2), Ca$^{2+}$-binding proteins (calmodulin (CaM) and TnC), and Ca$^{2+}$-dependent enzymes (myosin light chain kinase (MLCK) and SR Ca$^{2+}$-ATPase) to these Ca$^{2+}$ transients. Previously investigators determined Ca$^{2+}$ on-rates by rapidly mixing Ca$^{2+}$ with an apo Ca$^{2+}$-binding protein and determined Ca$^{2+}$ off rates by mixing a Ca$^{2+}$ chelator with a Ca$^{2+}$-saturated Ca$^{2+}$-binding protein. Our new approach allows us to follow the consequences of both Ca$^{2+}$ binding and Ca$^{2+}$ removal including: Ca$^{2+}$-induced conformational changes; Ca$^{2+}$-induced protein/peptide and protein/protein interactions; and enzyme activation and inactivation, in response to Ca$^{2+}$ transients of various amplitude and duration. This has enabled us to define how these Ca$^{2+}$-binding proteins and Ca$^{2+}$-dependent enzymes are tuned to receive and transduce the Ca$^{2+}$ signal into cellular actions.
CaM and more than 400 other Ca\textsuperscript{2+}-binding proteins use the EF-hand motif to bind Ca\textsuperscript{2+} and/or Mg\textsuperscript{2+}. The acid pair hypothesis proposes that the number and location of negatively charged residues positioned on the axes of the Ca\textsuperscript{2+}-binding loop of an EF-hand can tune the affinity of Ca\textsuperscript{2+}/Mg\textsuperscript{2+} binding. These residues in the Ca\textsuperscript{2+}-binding loop are geometrically arranged on the axes of a pentagonal bipyramid at positions 1 (+X), 3 (+Y), 5 (+Z), 7 (-Y), 9 (-X) and 12 (-Z). We have used site-directed mutagenesis to change the number and location of acidic residues paired on the X (+X, -X positions), Y (+Y, -Y positions) and Z (+Z, -Z positions) axes, called acid pairs, in CaM's two N-terminal EF-hands. This produced a series of mutant CaM's with ∼560 and 430-fold differences in N-terminal Ca\textsuperscript{2+} and Mg\textsuperscript{2+} affinities, respectively. In order to increase Ca\textsuperscript{2+} affinity significantly to CaM's N-terminal paired EF-hand system when one of the EF-hands (site II) was functionally impaired, three acid pairs were required in the other EF-hand (site I). When both of CaM's N-terminal paired EF-hands were functional, only a single acid pair in either EF-hand was required to significantly increase Ca\textsuperscript{2+} affinity. An Z acid pair was required for high Mg\textsuperscript{2+} affinity in site I of CaM. The increases in both Ca\textsuperscript{2+} and Mg\textsuperscript{2+} affinity were primarily due to increases in the cation association rates. These studies also demonstrate that mutants with the widest range in N-terminal Ca\textsuperscript{2+} affinity exhibited only an ∼3-fold difference in Ca\textsuperscript{2+}-dependent activation of a CaM target enzyme.
Dedicated to my Loving Wife
ACKNOWLEDGMENTS

I wish to thank my advisor, Dr. J. David Johnson, for his invaluable guidance, support, encouragement, patience and freedom he gave me in achieving my Ph.D.

I can not begin to thank enough my loving wife for all her support, devotion and dedication that without, this Ph.D. would not have been possible.

I wish to thank Dr. Charles Brooks, Dr. Pal Vaghy and my committee members Dr. Jack Rall, Dr. Peter Reiser and Dr. Ruth Altschuld for their invaluable advice and scientific knowledge.

I wish to thank my colleagues: Ritsu Kondo, D.J. Black, Svetlana Tikonova, Christopher Snyder, Yangdong Jiang, Maera Flynn, and Ryan Rehl for their support and encouragement throughout my graduate studies.

I also thank my three children, Jordan, Grace and Oliver for all their hugs and kisses to ease a hard day. I also wish to thank my family, especially my mother and father, for their love, help, encouragement and support.
VITA

April 22, 1971 ..................................... Born - Ann Arbor, MI

1993 ................................................... B.A. Mathematics and Physics
Minor, Philosophy and Religion
Greenville College, Greenville, IL

1994 - present ..................................... Graduate Research Associate
Dept. Medical Biochemistry
The Ohio State University

PUBLICATIONS

Research Publications

   and Johnson, J.D. Reciprocal regulation of mammalian nitric oxide synthase and
calcineurin by plant calmodulin isoforms. (1998) Biochem. 37, 15593-15597

2. Wang, S., George, S. E., Davis, J. P. and Johnson, J. D. Structural determinants
   of Ca^{2+} exchange and affinity in the C terminal of cardiac troponin C. (1998)
   Biochem. 37, 14539 - 14544

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   (Ca) transients for the study of Ca exchange with Ca-binding proteins. (1996)
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FIELDS OF STUDY

Major Field: Biophysics
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>x</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xi</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>xiii</td>
</tr>
<tr>
<td>Chapters:</td>
<td></td>
</tr>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1 The calcium signal</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Troponin C and skeletal muscle contraction</td>
<td>6</td>
</tr>
<tr>
<td>1.3 Calmodulin</td>
<td>11</td>
</tr>
<tr>
<td>1.3.1 Brief introduction</td>
<td>11</td>
</tr>
<tr>
<td>1.3.2 General structure</td>
<td>13</td>
</tr>
<tr>
<td>1.3.3 Calmodulin as a calcium sensor</td>
<td>15</td>
</tr>
<tr>
<td>1.3.4 Peptide recognition</td>
<td>16</td>
</tr>
<tr>
<td>1.3.5 Target protein activation</td>
<td>20</td>
</tr>
<tr>
<td>1.3.6 Calcium binding properties</td>
<td>22</td>
</tr>
<tr>
<td>1.4 The EF-hand</td>
<td>22</td>
</tr>
<tr>
<td>1.4.1 Brief introduction</td>
<td>22</td>
</tr>
<tr>
<td>1.4.2 General structure</td>
<td>23</td>
</tr>
<tr>
<td>1.4.3 Calcium affinity</td>
<td>26</td>
</tr>
<tr>
<td>1.4.4 Calcium exchange rates</td>
<td>30</td>
</tr>
<tr>
<td>1.4.5 Cation selectivity</td>
<td>32</td>
</tr>
</tbody>
</table>
4.2.8 Ca\(^{2+}\)-dependent activation of phosphodiesterase by Ca\(^{2+}\)-binding site I and II acid pair mutants.........................................................107

4.3 Discussion................................................................................................110

4.4 Conclusion................................................................................................122

4.5 Future experiments..................................................................................123

Appendix..............................................................................................................126

5.1 Computer modeling of the transient occupancy of TnC.........................126

5.2 Computer modeling of the N-terminal of CaM binding to MLCK following ACTs of various duration.........................................................129

References............................................................................................................132
LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Most common amino acids found in EF-hand Ca$^{2+}$-binding sequences</td>
</tr>
<tr>
<td>3.1</td>
<td>Comparison of the association rates, dissociation rates and affinities for Ca$^{2+}$ indicators and proteins exposed to ACTs calculated or measured for 10°C</td>
</tr>
<tr>
<td>4.1</td>
<td>Comparison of the Ca$^{2+}$ affinities for site I acid pair mutants with an inactivated site II</td>
</tr>
<tr>
<td>4.2</td>
<td>Comparison of the Ca$^{2+}$ affinities, dissociation rates and association rates of the site I and site II acid pair mutants</td>
</tr>
<tr>
<td>4.3</td>
<td>Comparison of the Mg$^{2+}$ affinities, dissociation rates and association rates of the W-site I acid pair mutants</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1 Cartoon schematic of calcium signaling</td>
<td>5</td>
</tr>
<tr>
<td>1.2 Structure of a skeletal muscle cell and its sarcomere</td>
<td>7</td>
</tr>
<tr>
<td>1.3 Troponin C regulation of striated muscle contraction</td>
<td>9</td>
</tr>
<tr>
<td>1.4 Calmodulin regulated proteins and enzymes</td>
<td>12</td>
</tr>
<tr>
<td>1.5 Crystal structure of calcium bound calmodulin</td>
<td>14</td>
</tr>
<tr>
<td>1.6 Crystal structure of calcium-calmodulin complexed with the calmodulin binding peptide from smooth muscle myosin light chain kinase</td>
<td>19</td>
</tr>
<tr>
<td>1.7 Target protein activation by calmodulin</td>
<td>21</td>
</tr>
<tr>
<td>1.8 Calcium coordination of calcium-binding site I of calmodulin</td>
<td>25</td>
</tr>
<tr>
<td>3.1 Response of the $\text{Ca}^{2+}$ indicators mag-fura-2 and fluo-3 to a 1.2 ms half width artificial $\text{Ca}^{2+}$ transient</td>
<td>43</td>
</tr>
<tr>
<td>3.2 Effect of [EGTA] on the half width of artificial $\text{Ca}^{2+}$ transients</td>
<td>46</td>
</tr>
<tr>
<td>3.3 Effect of [Mg$^{2+}$] on the half width of artificial $\text{Ca}^{2+}$ transients created with EDTA</td>
<td>49</td>
</tr>
<tr>
<td>3.4 $\text{Ca}^{2+}$ occupancy of the N-terminal $\text{Ca}^{2+}$ specific regulatory sites of troponin C during artificial $\text{Ca}^{2+}$ transients of different duration</td>
<td>53</td>
</tr>
<tr>
<td>3.5 The time course of $\text{Ca}^{2+}$ and of RS-20 dissociation from the $\text{Ca}^{2+}$-CaM-RS-20 complex</td>
<td>55</td>
</tr>
<tr>
<td>3.6 The time course of the decrease in $\text{Ca}^{2+}$-CaM-RS-20 Trp fluorescence upon $\text{Ca}^{2+}$ chelation by EGTA and after a 1.1 ms half width artificial $\text{Ca}^{2+}$ transient</td>
<td>58</td>
</tr>
</tbody>
</table>
3.7 The binding of CaM-MIANS to MLCK during artificial Ca\(^{2+}\) transients of different duration.....................................................................................61

3.8 Time course of activation of the SR Ca\(^{2+}\)-ATPase by artificial Ca\(^{2+}\) transients of different duration.....................................................................................65

4.1 Ca\(^{2+}\) binding to site I acid pair mutants with an inactivated site II..............82

4.2 Ca\(^{2+}\) binding to site I acid pair mutants with a functional Ca\(^{2+}\)-binding site II...........................................................................................................87

4.3 Ca\(^{2+}\) binding to W-site I acid pair mutants with a functional Ca\(^{2+}\)-binding site II...............................................................................................89

4.4 Comparison of Ka values for the site II acid pair mutants with a functional site I.................................................................93

4.5 Ca\(^{2+}\) dissociation from site I acid pair mutants with a functional site II........97

4.6 Ca\(^{2+}\) dissociation from the W-site I acid pair mutants with a functional site II.........................................................................................................98

4.7 Comparison of the Ca\(^{2+}\) association rates of the site I acid pair mutants with a functional site II.................................................................99

4.8 Mg\(^{2+}\) binding to site I acid pair mutants with a functional site II.................102

4.9 Mg\(^{2+}\) dissociation from W-site I acid pair mutants with a functional site II...............................................................................................104

4.10 Comparison of the Mg\(^{2+}\) dissociation and association rates for the W-site I acid pair mutants with a functional site II........................................106

4.11 Ca\(^{2+}\)-dependent activation of Phosphodiesterase by site I and site II acid pair mutants........................................................................................................109

5.1 Computer simulation of the Ca\(^{2+}\) occupancy of the N-terminal Ca\(^{2+}\) specific regulatory sites of troponin C during artificial Ca\(^{2+}\) transients of different duration...........................................................................................................128

5.2 The binding of the N-terminal of CaM to MLCK during artificial Ca\(^{2+}\) transients of different duration.....................................................................................131
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT</td>
<td>Artificial Ca transient</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaM</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>CaMKII</td>
<td>CaM-dependent protein kinase II peptide (281-309) (MHRQETVDCLKKPNARRKLKGAILTTMLA)</td>
</tr>
<tr>
<td>DANZ</td>
<td>Dansylaziridine</td>
</tr>
<tr>
<td>DiR</td>
<td>Dihydropyridine receptor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>hw</td>
<td>Half width</td>
</tr>
<tr>
<td>I</td>
<td>First N-terminal Ca-binding site in CaM</td>
</tr>
<tr>
<td>II</td>
<td>Second N-terminal Ca-binding site in CaM</td>
</tr>
<tr>
<td>IP3R</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>M-13</td>
<td>Skeletal muscle myosin light chain kinase peptide (KRRWKKNFIAVSAANRFKKISSSGAL)</td>
</tr>
<tr>
<td>MF2</td>
<td>Mag-fura-2</td>
</tr>
<tr>
<td>MIANS</td>
<td>2-(4'-maleimidoanilino)-naphthalene-6-sulfonic acid</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>Mops</td>
<td>3-(N-Morpholino)-propanesulfonic acid</td>
</tr>
<tr>
<td>Pi</td>
<td>Inorganic phosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PSI</td>
<td>Pseudo-substrate inhibitory domain</td>
</tr>
<tr>
<td>RS-20</td>
<td>Smooth muscle myosin light chain kinase peptide (796-815) (ARRKWQKTGHAVRAIGRLSS)</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic Reticulum</td>
</tr>
<tr>
<td>T-tubules</td>
<td>Transverse tubules</td>
</tr>
<tr>
<td>TBQ</td>
<td>2,5-di-(tert-butyl)-1,4-benzohydroquinone</td>
</tr>
<tr>
<td>TnC</td>
<td>Troponin C</td>
</tr>
<tr>
<td>TnI</td>
<td>Troponin I</td>
</tr>
<tr>
<td>TNP-ATP</td>
<td>2',3'-O-(2,4,6-trinitrophenyl)-ATP</td>
</tr>
<tr>
<td>TnT</td>
<td>Troponin T</td>
</tr>
<tr>
<td>W</td>
<td>CaM mutant containing F19W</td>
</tr>
<tr>
<td>wt</td>
<td>Wild type</td>
</tr>
<tr>
<td>X</td>
<td>X acid pair in a EF-hand Ca-binding loop</td>
</tr>
<tr>
<td>Y</td>
<td>Y acid pair in a EF-hand Ca-binding loop</td>
</tr>
<tr>
<td>Z</td>
<td>Z acid pair in a EF-hand Ca-binding loop</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 The Calcium Signal

As you sit to read this dissertation, you breath, your heart beats, many of your muscles move, you may be digesting the remnants of a good meal, and possibly your head already aches. All of these phenomena would not occur had not there been a change in your various cell types’ intracellular [Ca\textsuperscript{2+}]. Almost everything that we do, or our body does to us, is controlled in one way or another by fluxes in [Ca\textsuperscript{2+}]. Thus, Ca\textsuperscript{2+} is an extremely important second messenger used by cells to regulate activity such as muscle contraction, neurotransmission, metabolism, secretion, gene expression, and protein synthesis (1). Numerous Ca\textsuperscript{2+} regulated systems are functioning from the moment of fertilization, throughout the development and growth of an organism, and in some cases, even lead to apoptotic events (2). The plethora of cellular functions controlled by Ca\textsuperscript{2+}, as well as their diverse nature, results from a flexible yet tightly regulated generation of cellular Ca\textsuperscript{2+} signals. These Ca\textsuperscript{2+} signals rise transiently to evoke the desired response and then fall, usually discontinuing the response.
Under resting conditions in a cell, $[\text{Ca}^{2+}]_{\text{free}}$ is typically maintained at $\sim 100$ nM. Cells can maintain this low $[\text{Ca}^{2+}]$ by actively pumping $\text{Ca}^{2+}$ into internal stores such as the sarcoplasmic or endoplasmic reticulum (SR and ER, respectively) or by extruding it across the plasma membrane (Figure 1.1). This is an amazing accomplishment considering that the $[\text{Ca}^{2+}]$ outside of a cell is $\sim 1.2$ mM (3) creating an $\sim 12,000$-fold concentration gradient to work against. The plasma membrane utilizes $\text{Ca}^{2+}$-ATPases and $\text{Na}^{+}/\text{Ca}^{2+}$ exchangers to keep the resting $[\text{Ca}^{2+}]$ low. The SR and ER also use $\text{Ca}^{2+}$-ATPases to sequester $\text{Ca}^{2+}$ inside these organelles. The SR and ER are able to hold large quantities of $\text{Ca}^{2+}$ due to the presence of specialized, high capacity, yet low affinity $\text{Ca}^{2+}$ buffering proteins within these compartments, such as calsequestrin and calreticulin. Furthermore, specific $\text{Na}^{+}$ and $\text{K}^{+}$ channels within the plasma membrane can regulate the electrical polarization of the cell to either increase or decrease $\text{Ca}^{2+}$ conductivity. All of these mechanisms work cooperatively to reduce or maintain low $[\text{Ca}^{2+}]$ within a cell.

There are two sources from which a cell can recruit and increase its $\text{Ca}^{2+}$ content. One source is from outside the cell, while the other source comes from the cell’s internal stores, such as the SR or ER. Depending on the demand for $\text{Ca}^{2+}$, and the cell type, each source can be utilized either together or independently. An increase in cellular $\text{Ca}^{2+}$ can be triggered by electrical stimulation and generation of an action potential, receptor binding of hormones or drugs, or by store-operated $\text{Ca}^{2+}$ release created when internal $\text{Ca}^{2+}$ stores are depleted.
In the case of an excitable cell (3), a change in membrane potential can activate voltage gated Ca\(^{2+}\) channels to cause a rise in intracellular Ca\(^{2+}\), as occurs in neurons and striated muscle cells. The plasma membrane can also contain receptor-operated Ca\(^{2+}\) channels that open and let Ca\(^{2+}\) into the cell upon binding of agonists, such as certain neurotransmitters in neurons. In the case of platelets, and certain types of non-excitatory cells (4), when internal Ca\(^{2+}\) stores are emptied a plasma membrane associated store-operated Ca\(^{2+}\) channel can open to allow Ca\(^{2+}\) to enter the cell. Thus, a cell has many options to allow the entry of Ca\(^{2+}\) across its plasma membrane. At times, the opening of plasma membrane Ca\(^{2+}\) channels can cause the release of Ca\(^{2+}\) from internal stores as well, termed Ca\(^{2+}\)-induced Ca\(^{2+}\) release.

A prime example of Ca\(^{2+}\)-induced Ca\(^{2+}\) release is observed in cardiac muscle. Cardiac muscle cells have a specialized plasma membrane system which invaginates into the cardiac fibers at the Z line, called transverse tubules (T-tubules). These T-tubules contain the dihydropyridine receptor (DiR), which is organized in a specific pattern close to the SR membrane’s ryanodine receptors (RyR). Both of these receptors are Ca\(^{2+}\) channels. Upon membrane depolarization, the DiR opens and releases Ca\(^{2+}\) in the vicinity of the RyR which then binds the Ca\(^{2+}\) causing release of Ca\(^{2+}\) from the SR’s internal pool of Ca\(^{2+}\). It is the release of Ca\(^{2+}\) from the SR that initiates contraction of the cardiac muscle. In other types of cells, the ER membrane can also contain another specialized Ca\(^{2+}\) release channel, called the inositol 1,4,5-trisphosphate receptor (IP\(_3\)R). This receptor releases Ca\(^{2+}\) from the ER upon binding of IP\(_3\). When hormones bind to certain types of G-protein-linked receptors or when
growth factors bind to receptor tyrosine kinases, specific isoforms of phospholipase C are activated to produce IP3, and cause release of Ca$^{2+}$. Cells utilize variations and combinations of Ca$^{2+}$ entry across the plasma membrane and release of Ca$^{2+}$ from internal stores to generate a Ca$^{2+}$ transient. These Ca$^{2+}$ transients occur in a defined space, with a particular amplitude and for a finite period of time. A prime example of the spatial effects of Ca$^{2+}$ release occurs in smooth muscle. Smooth muscle contraction can be initiated when multiple Ca$^{2+}$ channels are activated to create a global cellular Ca$^{2+}$ signal. Although, when Ca$^{2+}$ is elevated only within the vicinity of the plasma membrane, a K+ channel can be activated which causes the smooth muscle to relax (5, 6). Thus, the space in which Ca$^{2+}$ is released can be of major importance to which activity the cell wants to control. The amplitude of the Ca$^{2+}$ transient is another key element that a cell will use to modify a specific activity. It has been shown in B lymphocytes that Ca$^{2+}$ transients of varying amplitude control the differential activation of specific transcriptional regulators (7). Furthermore, the duration of the Ca$^{2+}$ transient can have marked effects on the duration and strength of a cellular response to Ca$^{2+}$. When the duration of the Ca$^{2+}$ transients were increased in frog skeletal muscle, there was a linear increase in the amount of force generated (8). Thus, the cell's ability to generate Ca$^{2+}$ transients in a particular location, with specific amplitude, and for designated periods of time appears to be a mechanism in which a cell can modify its different activities with the same messenger, Ca$^{2+}$. This would imply that there are receivers within the cell that are tuned precisely to bind Ca$^{2+}$ only during a Ca$^{2+}$ transient that matches their
binding parameters. One of the most studied and understood Ca$^{2+}$-dependent systems is skeletal muscle contraction.

Figure 1.1: Cartoon schematic of calcium signaling.

A rise in the calcium transient can be caused by either Ca$^{2+}$ entering the cell from the extracellular space or release from internal stores, such as the SR. Different types of cells will utilize various Ca$^{2+}$ channels and receptor operated IP$_3$ generating schemes to elevate cytosolic Ca$^{2+}$. Depending on the electrical potential of the cell (controlled by specific monovalent cation channels) and the activity demand of the cell, the amplitude, duration and region in which the Ca$^{2+}$ is elevated can vary. Eventually, the cell must remove the Ca$^{2+}$ from the cytosol. This task is accomplished by Ca$^{2+}$-ATPases within the plasma membrane and SR, and by cation exchangers, such as the Na$^+$/Ca$^{2+}$ antiporter.
1.2 Troponin C and Skeletal Muscle Contraction

The skeletal muscle cell contains many interdigitating myofilaments consisting primarily of two types of filaments, termed thick and thin (Figure 1.2). The thick filament contains predominantly myosin, while the thin filament contains actin, tropomyosin and the troponin complex. Three proteins make up the troponin complex: the Ca$^{2+}$-binding subunit, troponin C (TnC); the inhibitory subunit, troponin I (TnI); and the tropomyosin-binding subunit, troponin T (TnT). The thick and thin filaments are packed in a highly ordered lattice, with a repeating functional unit called the sarcomere.

Skeletal muscle has an electrically excitable plasma membrane, called the sarcolemma that also forms T-tubules that invaginate the muscle fiber. As was the case with cardiac muscle, within the T-tubules are the DiR. In skeletal muscle the DiR makes physical contact with the RyR of the SR. When the action potential is propagated down the T-tubule the DiR acts as a voltage sensor through structural changes in the protein to trigger release of Ca$^{2+}$ from the RyR resulting in an ~10 μM step-increase in [Ca$^{2+}$] (9).
Figure 1.2: Structure of a skeletal muscle cell and its sarcomere.

The sarcolemma membrane surrounds the muscle cell and forms T-tubules which invaginate deep into the muscle fiber close to the SR network. The sarcomere is the functional repeating unit within the muscle cell. Thick (myosin containing) and thin (actin containing) filaments overlap in the sarcomere and slide past one another to generate force.
When \([\text{Ca}^{2+}]\) is elevated after an action potential, TnC's rapid N-terminal regulatory \(\text{Ca}^{2+}\)-binding sites bind \(\text{Ca}^{2+} (-1 \times 10^8 \text{ M}^{-1}\text{s}^{-1})\) (10). The binding of \(\text{Ca}^{2+}\) to TnC causes conformational changes within the thin filament such that TnI no longer inhibits the interaction of myosin with actin (Figure 1.3). Myosin can then bind to actin and form a cross-bridge. Through the energy of ATP hydrolysis and conformational changes within myosin, the thick and thin filaments slide past one another causing tension in the muscle. As long as TnC is bound to \(\text{Ca}^{2+}\) myosin and actin can cyclically interact.

Upon binding of ATP, myosin's affinity for actin decreases. However, ATP is quickly hydrolyzed to form a myosin-ADP-P\(_i\) complex which has high affinity for actin. The myosin-ADP-P\(_i\) complex then binds actin forming a new cross-bridge. Subsequently, the P\(_i\) leaves the cross-bridge complex and a power stroke results, sliding the thin and thick filaments past one another. Before the crossbridge can be broken, ADP must dissociate from the complex and myosin must rebind another ATP molecule. Once this occurs, myosin-ATP dissociates from actin and the cycle can repeat itself until ATP is depleted or the \(\text{Ca}^{2+}\) signal subsides.
Upon binding of Ca$^{2+}$ to the N-terminal regulatory sites of troponin C, structural changes occur in the troponin complex which relieve the inhibition of myosin interacting with actin. Once this occurs, the muscle is able to generate force, through the cyclic interactions of actin and myosin crossbridges. When the Ca$^{2+}$ signal subsides, Ca$^{2+}$ dissociates from troponin C and myosin is inhibited from interacting with actin, and the muscle relaxes.
Typically, the excitatory stimulus on the muscle is removed and the SR stops dumping Ca\(^{2+}\) into the cytosol due to the inhibition of the RyR by binding of Ca\(^{2+}\)-calmodulin (11). Once this occurs, the SR-Ca\(^{2+}\)-ATPases are able to remove Ca\(^{2+}\) from the cytosol eventually bringing the \([\text{Ca}^{2+}]_{\text{free}}\) below the Ca\(^{2+}\) affinity of the N-terminal Ca\(^{2+}\)-binding sites of TnC. In some skeletal muscle cells there is another specialized Ca\(^{2+}\) buffering protein, parvalbumin. Parvalbumin aids the SR by decreasing the \([\text{Ca}^{2+}]\) within the muscle cell. It does so by slowly exchanging Mg\(^{2+}\) for Ca\(^{2+}\) (~ 3 s\(^{-1}\)) at its Ca\(^{2+}\)-binding sites, which eventually is released for the SR-Ca\(^{2+}\)-ATPases to sequester (12). Thus, parvalbumin, unlike the N-terminal of TnC, binds Ca\(^{2+}\) slowly and with high affinity. Ca\(^{2+}\) removal by the SR and parvalbumin cause Ca\(^{2+}\) to dissociate from TnC. Subsequently, TnI's inhibitory function is restored and the interactions of myosin and actin cease. With all of these steps completed, the muscle relaxes. Thus, Ca\(^{2+}\) regulates the contractile state of skeletal muscle through the rapid N-terminal Ca\(^{2+}\)-binding sites of TnC. While TnC is a tissue specific Ca\(^{2+}\) sensor, calmodulin (CaM), is a ubiquitous eukaryotic Ca\(^{2+}\) sensor found in the same protein family as TnC.
1.3 Calmodulin

1.3.1 Brief Introduction

CaM was first discovered independently in the early 1970's by Cheung (13) and Kakiuchi and Yamazaki (14) as the activating factor of cyclic nucleotide phosphodiesterase (PDE). Subsequently, Teo and Wang (15) showed that the activating factor of PDE (CaM) required Ca\(^{2+}\). It soon became apparent that CaM was the activator of numerous other target enzymes and proteins which required Ca\(^{2+}\). Currently, CaM has been shown to activate or modulate more than 30 different enzymes and proteins typically in a Ca\(^{2+}\)-dependent manner (Figure 1.4). CaM can be thought of as a molecular switch that is turned “on” by cellular Ca\(^{2+}\) signals, and turned “off” by a subsequent fall in cellular Ca\(^{2+}\). Cells use Ca\(^{2+}\)-CaM to regulate diverse cellular activities such as smooth muscle contraction, neuronal signaling, Ca\(^{2+}\) homeostasis and cell cycle progression to list a few (16). Ultimately CaM supports life itself, since the loss of the CaM gene was fatal as demonstrated in yeast by Davis et al. (17) and in Drosophila by Nelson et al. (18).
Figure 1.4: Calmodulin regulated proteins and enzymes.

CaM regulates more than 30 different enzymes through its binding of Ca\(^{2+}\). CaM can regulate the levels of cyclic nucleotides through cyclases and phosphodiesterases. Ca\(^{2+}\) homeostasis can be regulated by CaM upon binding Ca\(^{2+}\)-ATPases. Phosphorylation events that modify cell activity can also be regulated by CaM stimulation of kinases and phosphatases. Another important second messenger, nitric oxide, is CaM regulated through nitric oxide synthase. CaM can bind to many cytoskeletal and nuclear proteins as well.
1.3.2 General Structure

CaM can be encoded by a single gene or by multiple genes which generate typically the identical amino acid sequence in mammalian systems (19, 20). It has been shown in plants that multiple isoforms of CaM can be generated by a family of CaM genes (21, 22). The significance of having multiple isoforms of CaM is not completely understood but is diligently being pursued (23). CaM is an acidic protein made of a single polypeptide chain of 148 amino acids with a molecular weight of ~16,700 Daltons. The crystal structure of Ca$^{2+}$ saturated CaM was first described by Babu et al. (24) and later refined to high resolution (1.7 Å) by Chattopadhyaya et al. (25).

The overall tertiary structure of CaM is that of a 65 Å long dumbbell, with 25 x 25 x 20 Å globular N- and C-terminal domains separated by a flexible 28 residue central α-helix (Figure 1.5). The secondary structure of CaM is composed primarily of eight helices and four Ca$^{2+}$-binding loops. The first four helices (named N-terminal to C-terminal A, B, C and D, respectively) are in the N-terminal domain of CaM and the last four helices (named E, F, G and H) are in the C-terminal domain. Helices A/B, C/D, E/F and G/H form individual helix-loop-helix domains within their respective terminals. The central helix of CaM is comprised of the D and E helices from the N- and C-terminals, respectively.
CaM is a dumbbell shaped protein with its N- and C-terminal globular domains connected by a 28 residue flexible central helix. Both domains of CaM contain two coupled EF-hand Ca$^{2+}$-binding motifs. Ca$^{2+}$ binding to CaM exposes hydrophobic pockets within each N- and C-terminal domain which allows CaM to bind and activate target enzymes. Figure adapted from Babu et al. (24).
The N- and C-terminals of CaM both bind two Ca\(^{2+}\) ions, one at each of the four helix-loop-helix repeats. Each of the repeating helix-loop-helix elements is a member of the EF-hand Ca\(^{2+}\)-binding motif family. The EF-hand will be discussed in greater detail and in a broader context later in this chapter. The loop domain of each EF-hand contains the structural elements necessary to bind Ca\(^{2+}\).

1.3.3 Calmodulin as a Calcium Sensor

CaM acts as a Ca\(^{2+}\) sensor because it has primarily two conformations, one in the apo state (off) and another in the Ca\(^{2+}\) saturated state (on). With the recent NMR structures of apo CaM solved (26, 27) it has been possible to see exactly how CaM can act as a molecular switch. Upon Ca\(^{2+}\) binding, the overall secondary structure of CaM changes very little, but the tertiary structures of both the N- and C-terminal domains change substantially. The N- and C-terminal domains of CaM adopt a "closed" conformation in the absence of Ca\(^{2+}\) such that, the four helices in each domain form a nearly antiparallel tight bundle with interhelical angles of approximately 180\(^\circ\). When CaM binds Ca\(^{2+}\) there is a substantial reordering of the packing of the helices within each domain causing the domains to "open" due to the angles between each paired helices becoming nearly perpendicular. These large structural rearrangements in helical packing upon Ca\(^{2+}\) binding expose large hydrophobic surfaces (~10 x 12.5 Å) in both the N- and C-terminal domains of CaM (24, 27). It is the exposure of both these hydrophobic pockets that turns CaM "on" and allows it to
interact with its target enzymes and proteins. Subsequent removal of Ca\(^{2+}\) from CaM reverses the aforementioned effects of binding Ca\(^{2+}\), and CaM again is turned “off.”

1.3.4 Peptide Recognition

The CaM recognition sequence of myosin light chain kinase (MLCK) was the first of the CaM target sequences to be identified (28, 29). Now there are many CaM binding domains described throughout the literature (30). Even though there is low sequence homology between CaM target binding domains, there are characteristics that most of the sequences share in common. For instance, the peptides composed of ~20 -30 amino acids typically can be represented on a helical wheel as basic, amphiphilic \(\alpha\)-helices such that basic and hydrophobic residues align on opposite faces of the helix. The sequences also tend to contain two bulky hydrophobic residues separated by twelve residues and located at opposite ends of the peptide. Often an Arg residue precedes the second bulky hydrophobic residue, as is the case for the smooth muscle MLCK CaM-binding peptide (RS-20). The hydrophobic residues of the CaM binding peptide associate with the exposed hydrophobic pockets of the N- and C-terminals of CaM, while the basic residues tend to interact with the solvent and acidic residues of CaM.

Currently there are no three-dimensional structures of CaM complexed to whole enzymes. However, there are structures available in which CaM is bound to target peptides derived from the CaM-binding domains of target enzymes. The first Ca\(^{2+}\)-CaM-peptide structure to be solved was with the 26-residue peptide corresponding to
the CaM-binding domain of skeletal muscle MLCK (M-13) (31). Soon afterwards, the crystal structures of Ca\textsuperscript{2+}-CaM complexed to the 20-residue RS-20 peptide and 24-residue CaM-II kinase peptide (CaMKII) were solved (32, 33).

There are common features between the three structures of the Ca\textsuperscript{2+}-CaM target peptide complexes. When CaM binds to the target peptides its overall structure shifts from the dumbbell shape to an ellipsoid shape in which the N- and C-terminals of CaM engulf the peptide and come in close proximity to one another, even forming contacts between helices B and G. This change in CaM’s structure is easily seen in the Ca\textsuperscript{2+}-CaM-RS-20 complex (Figure 1.6). In order for CaM to achieve this structure, the central helix of CaM unwinds between residues 73-77 allowing the N- and C-terminals to bend 100° and twist 120° with respect to one another. This large structural change in CaM causes the exposed hydrophobic pockets in the N- and C-terminals of CaM to create a channel which accommodates the peptide. The peptide anchors itself in the hydrophobic channel with its N-terminal bound to the C-terminal of CaM and its C-terminal bound to the N-terminal of CaM by its two bulky hydrophobic residues. Interestingly, even though the N- and C-terminal domains of CaM have engulfed the peptide, their overall domain structures are basically unaffected by the peptide binding event.

The major difference between the structures stem from the fact that CaMKII has only eight residues between its two bulky hydrophobic residues instead of twelve (32, 33). In order for CaM to bind the shorter peptide of CaMKII the central helix unwinds further between residues 73 and 83 allowing the N- and C-terminal domains
of CaM to be closer to one another than they were in the two MLCK complexes (33). Thus, one mechanism by which CaM can recognize and bind to the many different CaM target enzymes is through the flexibility of the central helix, which was originally proposed by Persechini and Kretsinger (34). Interestingly, most of the contacts made between the peptides and CaM are Van der Walls contacts, which tend to be less specific as opposed to the more specific requirements of hydrogen bonds. Thus, the nonspecific binding of CaM to peptides may be another mechanism that allows CaM to interact with a large array of target sequences.
Figure 1.6: Crystal structure of calcium-calmodulin complexed with the calmodulin binding peptide from smooth muscle myosin light chain kinase.

The binding of Ca$^{2+}$ to CaM exposes hydrophobic pockets in both its N- and C-terminal domains which allow CaM to bind basic amphiphilic $\alpha$-helical peptides. The outer diameter circular protein is CaM wrapped around RS-20. CaM has to bend and twist in order to complex the enzyme. CaM is able to bind to different target peptides and enzymes due to the flexibility of CaM's central helix (32).
1.3.5 Target Protein Activation

Often the CaM-binding domains of CaM-dependent enzymes are located either near or within a sequence of the enzyme called a pseudo-substrate inhibitory domain (PSI) (Figure 1.7). The PSI is a region of the enzyme that is homologous to the region of the substrate where the enzyme binds. Thus, the PSI inhibits the enzyme by blocking its active site from binding substrate protein(s). This mechanism of autoinhibition is observed in skeletal and smooth muscle MLCK, CaM II kinase, calcineurin, Ca\(^{2+}\)-ATPase and phosphorylase kinase (35 - 40). These enzymes can be made constitutively active by cleaving the PSI from the enzyme (41) and then inhibited again by addition of the PSI peptide (35, 36). The autoinhibited enzymes can also be activated by the binding of Ca\(^{2+}\)-CaM. When Ca\(^{2+}\) binds CaM, it is able to bind the PSI domain, removing it from the active site of the enzyme which facilitates enzyme activation. Upon Ca\(^{2+}\) removal from the cell, Ca\(^{2+}\) dissociates from CaM and CaM then dissociates from the enzyme. Once this occurs, the PSI domain can then auto-inhibit the enzyme again. Thus, the removal of a PSI domain is one mechanism in which CaM can activate and regulate target enzymes.
Figure 1.7: Target protein activation by calmodulin. A PSI domain, homologous to the substrate binding sequence, autoinhibits the enzyme by blocking substrate binding.

Overlapping, or adjacent to the PSI domain is a CaM-binding domain. When CaM binds Ca^{2+}, hydrophobic pockets in the N- and C-terminals of CaM are exposed which allow CaM to bind target peptides and enzymes. The binding of CaM to the enzyme removes the PSI domain from the enzyme’s active site, which allows it to interact with substrate.
1.3.6 Calcium Binding Properties

Critical to CaM activation of enzymes, as discussed above, is Ca$^{2+}$ binding to both its N- and C-terminal domains. The N-terminal Ca$^{2+}$-binding sites of CaM have ~3-6-fold lower Ca$^{2+}$ affinity (~3 - 10 μM) than the C-terminal Ca$^{2+}$-binding sites (~1 - 3 μM) (42, 43). The lower Ca$^{2+}$ affinity of the N-terminal of CaM is reflected in the fact that it has an approximately 80-170-fold faster Ca$^{2+}$ dissociation rate (43 - 45), but with an approximately 70-fold faster Ca$^{2+}$ association rate (43). Since the N-terminal of CaM has faster Ca$^{2+}$ binding and dissociation kinetics, it may be the regulatory domain of CaM similar in function to the N-terminal of TnC (43).

1.4 The EF-hand

1.4.1 Brief Introduction

The Ca$^{2+}$-binding motif in CaM and found in over 400 other proteins is the EF-hand. The EF-hand was first described by Kretsinger and Nockolds (46) in the crystal structure of Carp parvalbumin. Currently there are 39 distinct subfamilies of EF-hand Ca$^{2+}$-binding proteins which contain from 2 to 8 EF-hand domains per protein, comprising over 1,700 different EF-hand sequences (47). Of the 39 subfamilies, only 15 have known functionality and fewer than 5% of these proteins have been characterized for their cation binding properties (9). It is not surprising that the EF-hand is one of the five most common protein motifs used by nature, since Ca$^{2+}$ is an extremely important second messenger (1 - 3). It is surprising that proteins which
utilize this small Ca\(^{2+}\)-binding motif exhibit diverse cation affinity, selectivity and exchange rates. Comparisons of the cation binding properties of EF-hand proteins, suggest that Ca\(^{2+}\) affinity can vary by 10\(^6\)-fold (nM to mM affinities) and Ca\(^{2+}\) dissociation and association rates can vary by 10\(^3\)-fold (9, 48, 49).

1.4.2 General Structure

The canonical EF-hand consists of a linear sequence of 29 amino acids with the first 9 forming an “E” α-helix, followed by a 12 residue Ca\(^{2+}\)-binding loop whose last three residues blend into a final 8 residue “F” α-helix. There are regions and functions of the EF-hand that are conserved throughout the subfamily. The E-helix typically contains four hydrophobic residues located at positions 2, 5, 6 and 9, while the F-helix contains hydrophobic residues at positions 1, 4, 5 and 8 (Table 1.1). The Ca\(^{2+}\)-binding loop domain of the EF-hand coordinates Ca\(^{2+}\) binding through six chelating residues which donate 7 oxygen atoms geometrically arranged on the axes of a pentagonal bipyramid at positions 1 (+x), 3 (+y), 5 (+z), 7 (-y), 9 (-x) and 12 (-z) (Figure 1.7). These chelating residues donate carbonyl, carboxyl or hydroxyl oxygen atoms to coordinate Ca\(^{2+}\) or Mg\(^{2+}\) binding. The most common amino acids that coordinate Ca\(^{2+}\) are acidic, located at positions 1, 3, 5, 9, and 12 in the Ca\(^{2+}\)-binding loop. Of the chelating residues, positions 7 (-y) and 9 (-x) are the most variable, while the 1st and 12th residues are almost invariably Asp and Glu, respectively. Residue 6 in the Ca\(^{2+}\)-binding loop is often Gly which is thought to permit a sharp bend in the Ca\(^{2+}\)-binding loop.
Table 1.1: Most common amino acids found in EF-hand Ca\(^{2+}\)-binding sequences.

540 non-redundant EF-hand sequences were analyzed for their amino acid frequency at all 29 residues. The amino acids at each position in the E-helix, Ca\(^{2+}\)-binding loop and F-helix represent the most frequently occurring amino acid with its percent of frequency below.
Figure 1.8: Calcium coordination of calcium-binding site I of calmodulin.

The large dark sphere represents a coordinated Ca$^{2+}$ ion in the first N-terminal Ca$^{2+}$-binding loop of CaM. Even though the Ca$^{2+}$-binding loops of CaM's four EF-hands are not identical in sequence, each loop coordinates Ca$^{2+}$ through seven oxygen atoms donated by six residues. The oxygen atoms are arranged on the axes of a pentagonal bipyramid at positions x, y, z, -y, -x and -z, respectively. The -x oxygen is donated by a water molecule bridged by T28. Otherwise, each residue directly donates an oxygen atom either through its sidechain carboxylate oxygen(s) or its backbone carbonyl. Figure created by Melanie R. Nelson, in W.J. Chazin's laboratory at the Scripps Research Institute and adapted from Eldik and Watterson (16).
Typically EF-hands occur in coupled pairs related to one another by an approximate two-fold rotation axis. When paired, the coupled EF-hand's Ca$^{2+}$-binding loops form an antiparallel β-sheet between corresponding residues 7 - 9 in both loop domains. Within this β-sheet, residue 8 is almost invariably occupied by Ile, Val or Leu, which strengthens the β-sheet interactions. Also, the conserved hydrophobic residues of the E- and F-helices in the first EF-hand are thought to interact with the corresponding hydrophobic residues in the second EF-hand to stabilize the coupled system.

1.4.3 Calcium Affinity

As pointed out above, there is a million-fold range in Ca$^{2+}$ affinities observed in EF-hand Ca$^{2+}$-binding proteins. The reason for this is that there appear to be many interacting and overlapping factors that control Ca$^{2+}$ affinity of an EF-hand such as: hydrophobicity of the helices and Ca$^{2+}$-binding loop; character of the coordinating oxygen atoms; number of acidic coordinating residues and their position; and character of non-coordinating amino acids inside and outside of the Ca$^{2+}$-binding loop (9, 48, 49). These factors that control Ca$^{2+}$ affinity have been observed in isolated EF-hand peptides and in whole Ca$^{2+}$-binding proteins.

Malik et al. (50) demonstrated that the isolated peptide corresponding to Ca$^{2+}$-binding loop II of skeletal TnC could bind Ca$^{2+}$ (Kd ~100 mM). Further addition of the flanking helices to the Ca$^{2+}$-binding loop increased Ca$^{2+}$ affinity ~10-fold. Reid et al. (51) demonstrated a similar result with a synthetic EF-hand corresponding to the
third Ca\textsuperscript{2+} binding site of skeletal TnC. In these studies, it was shown that addition of
the F-helix to the Ca\textsuperscript{2+} binding loop had little effect on Ca\textsuperscript{2+} affinity compared to that
of the Ca\textsuperscript{2+}-binding loop alone, while addition of the E-helix increased Ca\textsuperscript{2+} affinity
\sim 1,250-fold. These studies suggest that the Ca\textsuperscript{2+}-binding loop is critical for Ca\textsuperscript{2+}
binding but the flanking helices are important factors that increase Ca\textsuperscript{2+} affinity of the
loop. Later, it was shown that Ca\textsuperscript{2+} binding to isolated EF-hand peptides created
dimers, which suggests the importance of an EF-hand pair in achieving high Ca\textsuperscript{2+}
affinity in a Ca\textsuperscript{2+}-binding protein (52, 53).

Within the context of the whole Ca\textsuperscript{2+}-binding protein, the nature of the residues
within the helices of EF-hands can also modify Ca\textsuperscript{2+} affinity. Monera et al. (54)
demonstrated that when buried, conserved hydrophobic residues within the E- and/or
F-helices in the third EF-hand of TnC were mutated to Ala the global Ca\textsuperscript{2+} affinity of
the C-terminal domain decreased \sim 100-fold. These studies imply that mutations that
remove the hydrophobic interactions between EF-hands destabilize the Ca\textsuperscript{2+} bound
state of a Ca\textsuperscript{2+}-binding protein and cause a decrease in Ca\textsuperscript{2+} affinity. Conversely, when
more hydrophobic residues were introduced into the hydrophobic core of calbindin D\textsubscript{9k}
which increased the thermal stability of the protein in the Ca\textsuperscript{2+}-bound state, the Ca\textsuperscript{2+}
affinity of the protein increased (55). Furthermore, Trigo-Gonzalez et al. (56) showed
that mutations (Ser and Thr) which increase the hydrogen bonding network and helical
content of an individual EF-hand helix increase Ca\textsuperscript{2+} affinity as well. These studies
demonstrate that mutations which stabilize the Ca\textsuperscript{2+} bound state of an EF-hand protein
through hydrophobic interactions and α-helical content of EF-hand helices are important factors which control Ca$^{2+}$ affinity.

The number and location of acidic residues which coordinate Ca$^{2+}$ are also important determinants of Ca$^{2+}$ affinity of EF-hand proteins. Almost invariably the $+x$ and $-z$ chelating residues in an EF-hand protein are Asp and Glu, respectively. When the $+x$ residue in the N-terminal of TnC was mutated to Glu, Ca$^{2+}$ binding to the N-terminal was annihilated (57). Studies have shown that mutation of Glu, at the $-z$ coordinating residue, to Asp, Gln, Lys or Ala practically annihilated Ca$^{2+}$ binding to the EF-hand which contained the mutation and decreased Ca$^{2+}$ affinity to the coupled EF-hand > 5-fold (57, 58). Thus, it would appear that high Ca$^{2+}$ affinity of an EF-hand is strictly dependent on the $+x$ and $-z$ positions being occupied by Asp and Glu, respectively. Interestingly, it has been recently proposed that the $-z$ coordinating residue in Ca$^{2+}$-binding site I in skeletal muscle TnC is the “triggering” residue that initiates the opening of the N-terminal hydrophobic pocket that ultimately leads to muscle contraction (59).

Reid and Hodges (60) proposed the acid pair hypothesis which relates the number and location of acidic residues at chelating positions in the Ca$^{2+}$-binding loop with Ca$^{2+}$ affinity. The acid pair hypothesis predicts that high Ca$^{2+}$ affinity should occur in a single EF-hand when there are maximally 4 acidic residues in chelating positions 1($+x$), 3($+y$), 5($+z$), 9($-x$) or 12($-z$). Furthermore, maximum Ca$^{2+}$ affinity should occur when the carboxylate oxygen atoms pair on the pentagonal bipyramid axes at the $+x$,$-x$ ($X$ acid pair) and $+z$,$-z$ ($Z$ acid pair) positions. The hypothesis
neglects the -y position since its coordination of Ca$^{2+}$ is through the peptide carbonyl oxygen and not the carboxylate sidechain.

The acid pair hypothesis has primarily been supported using synthetic EF-hand peptides. For instance, the third EF-hand of CaM has three acidic chelating residues and no acid pairs. It was shown that Ca$^{2+}$ affinity increased ~12-fold when the number of acid pairs was increased from zero to one in a synthetic peptide corresponding to the third Ca$^{2+}$-binding site of CaM (61). Furthermore, increasing the number of acid pairs to two (X and Z acid pairs) in this EF-hand peptide increased Ca$^{2+}$ affinity ~39-fold. These studies demonstrate that the number and location of Ca$^{2+}$ coordinating residues are important determinants of Ca$^{2+}$ affinity in an EF-hand.

The acid pair hypothesis has recently been tested in whole EF-hand Ca$^{2+}$-binding proteins. For instance, Ca$^{2+}$ affinity of the third Ca$^{2+}$-binding site of CaM was increased ~160-fold upon introduction of an X and Z acid pair (62). These studies were done in the presence of a mutation that inactivated Ca$^{2+}$ binding to the fourth Ca$^{2+}$-binding site of CaM, which also decreased Ca$^{2+}$ binding to the third site by ~24-fold compared to the parent CaM mutant. Wang et al. (63) demonstrated that Ca$^{2+}$ affinity could be increased ~3-fold when the X and Z acid pairs were incorporated into the third Ca$^{2+}$-binding site of CaM with a non-mutated fourth Ca$^{2+}$ binding site. The Ca$^{2+}$-binding protein oncomodulin contains an X acid pair in its first EF-hand and a Z acid pair in its second EF-hand. Ca$^{2+}$ affinity was increased >10-fold in oncomodulin by adding a Z acid pair in its first EF-hand or by adding an X acid pair in its second
EF-hand (64). Thus, increasing the number of acid pairs in a coupled EF-hand system can increase Ca\(^{2+}\) affinity.

Charged residues exposed to the solvent near an EF-hand’s Ca\(^{2+}\)-binding site can also modify Ca\(^{2+}\) affinity. In calbindin D\(_{\alpha}\), Ca\(^{2+}\) affinity decreased ~50-fold when negative surface charges, clustered around the Ca\(^{2+}\)-binding domain, were mutated to neutral residues (65). These acidic surface residues do not directly chelate the Ca\(^{2+}\) ion but may cause an increase in Ca\(^{2+}\) affinity by forming favorable ionic interactions with the solvent or other residues within the protein to stabilize the Ca\(^{2+}\)-bound state of the protein. In general, many factors contribute to an EF-hand’s Ca\(^{2+}\) affinity.

1.4.4 Calcium Exchange Rates

Ca\(^{2+}\) affinity (Kd) of a protein is determined by its Ca\(^{2+}\) association rate and dissociation rate (Kd = dissociation rate / association rate). Thus, changes in Ca\(^{2+}\) affinity must occur due to changes in Ca\(^{2+}\) kinetics. It was shown that the decrease in Ca\(^{2+}\) affinity caused by the loss of negative surface charges in the mutant calbindin D\(_{\alpha}\) was due to a ~50-fold decrease in the Ca\(^{2+}\) association rate (66). As mentioned earlier, when the Ca\(^{2+}\) bound state of calbindin D\(_{\alpha}\) was stabilized by substitutions that increased hydrophobic core interactions, Ca\(^{2+}\) affinity increased, caused by a nearly identical decrease in the Ca\(^{2+}\) dissociation rate (55). Furthermore, when the hydrophobic core of calbindin D\(_{\alpha}\) was destabilized by addition of less hydrophobic residues, the Ca\(^{2+}\) dissociation rate increased proportionately to the decrease in Ca\(^{2+}\)
affinity. These studies show that changes in Ca\(^{2+}\) affinity are manifested in changes in the Ca\(^{2+}\) association rate and/or the Ca\(^{2+}\) dissociation rate.

Before an EF-hand can bind Ca\(^{2+}\) it must first remove the hydration shell (seven water molecules) from around the cation. The calbindin studies suggest that negative surface charges near the Ca\(^{2+}\)-binding domain of an EF-hand can aid in the dehydration of the Ca\(^{2+}\), facilitating the binding process. On the other hand, Ca\(^{2+}\) dissociation rates can be tuned by the level of hydrophobic and ionic interactions that lead to the stabilization of the Ca\(^{2+}\)-bound protein. For example, a less stable Ca\(^{2+}\)-protein complex should have a faster Ca\(^{2+}\) dissociation rate than a more stable Ca\(^{2+}\)-protein complex.

Renner et al. (67) proposed an alternative explanation for control of Ca\(^{2+}\) binding kinetics to EF-hand proteins, called the gateway hypothesis. It would appear from the crystal and NMR structures of EF-hand proteins that the shortest pathway for a cation to bind to an EF-hand Ca\(^{2+}\)-binding loop is blocked by the -x chelating residue within the loop. This residue has been called the gateway residue, since it may serve as a "gate" that controls cation association and dissociation rates. The gateway hypothesis can be described as follows: 1) When the gateway sidechain has a neutral charge, shorter sidechains increase both the cation association and dissociation rates with little change in cation affinity. Longer sidechains are proposed to decrease both the cation association and dissociation rates with little change in cation affinity; 2) When the gateway sidechain was Asp, the cation dissociation rate decreased while the cation association rate increased, causing increased cation affinity; and 3) When the
gateway residue was Glu, the cation association rate was unchanged, but the cation
dissociation rate decreased causing increased cation affinity compared to the native
Gln residue. These observations were supported by studies in the galactose binding
protein which contains a single EF-hand-like Ca\(^{2+}\)-binding motif. This motif has the
same geometry of Ca\(^{2+}\) coordination like an EF-hand but its chelating residues are not
continuous in the primary sequence of the protein. Thus, conclusions drawn from
these studies should be taken cautiously. Furthermore, the studies used Tb\(^{3+}\) and not
Ca\(^{2+}\) as the cation. As has been shown by the same lab, mutations to the galactose
binding protein can affect trivalent cations in different ways than divalent cations (68).

1.4.5 Cation Selectivity

Certain EF-hand proteins must be able to respond to a rapid rise in Ca\(^{2+}\) and are
typically Ca\(^{2+}\) specific. A prime example of a Ca\(^{2+}\) specific EF-hand system is the N-
terminal Ca\(^{2+}\)-binding sites of skeletal TnC. These sites exhibit a Ca\(^{2+}\) affinity of ~3
\(\mu\)M and a Mg\(^{2+}\) affinity of ~5 mM, which is well above the [Mg\(^{2+}\)] \textit{in vivo} (~1mM)
(10). On the other side of the protein, the C-terminal Ca\(^{2+}\) binding sites of skeletal
TnC are considered Ca\(^{2+}\)/Mg\(^{2+}\) sites (Kd for Ca\(^{2+}\) of ~50 nM) since their affinity for
Mg\(^{2+}\) of ~200 \(\mu\)M implies that these sites will be bound with Mg\(^{2+}\) under resting
conditions in a cell. Typically EF-hand proteins that bind Ca\(^{2+}\) with high affinity also
bind Mg\(^{2+}\), albeit with \(~1 x 10^4\) lower affinity.

CaM has been shown to be able to bind spherical cations that have a radius
similar to that of Ca\(^{2+}\) (69). Thus, it would appear that CaM is able to discriminate
cations based on a size selectivity. The reason for this may be that the tertiary
structure of an EF-hand protein may be optimized in such a way that it can only accept cations of a particular size. If the cation is too small then the coordinating ligands cannot pack around the ion sufficiently to keep the ion bound. On the other hand, when a cation is too large, the constraints placed on the protein may not allow it to expand enough to accommodate the cation. Thus, the flexibility of an EF-hand may determine its cation selectivity (9). Another constraint placed on an EF-hand is charge selectivity (9). It is thought that the negative charges of the chelating residues in an EF-hand \( \text{Ca}^{2+} \)-binding loop create a charge repulsion on one another. Thus, in order for a cation to optimally bind to an EF-hand it must be able to overcome this charge repulsion. Monovalent ions such as \( \text{Na}^+ \) and \( \text{K}^+ \) bind to EF-hands with very low affinity possibly due to the fact that their positive charge is not great enough to overcome the negative charge repulsion of the EF-hand binding cavity. The coordination number of a cation may also play a role in cation binding.

\( \text{Ca}^{2+} \) is the only divalent cation that prefers a seven-fold coordination (9). \( \text{Mg}^{2+} \) requires a six-fold coordination. Thus, the seven-fold coordination sphere of an EF-hand protein is pre-formed to prefer \( \text{Ca}^{2+} \). The coordination number of an EF-hand can change to meet the requirements of \( \text{Mg}^{2+} \) binding. Parvalbumin has been crystallized in the presence of both \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) (70, 71). These studies show that \( \text{Ca}^{2+} \) binding to parvalbumin is of the typical 7-fold coordination, with the -z residue (Glu) donating two oxygen atoms. When \( \text{Mg}^{2+} \) binds parvalbumin the -z residue rotates so that only a single oxygen of the sidechain is involved with coordination and thus meets the requirements of 6-fold \( \text{Mg}^{2+} \) coordination. Thus, the flexibility of the -z
residue may be an important element of Mg$^{2+}$ binding. EF-hand peptide studies have shown that when an EF-hand contains a Z acid pair, Mg$^{2+}$ binding can occur, but without the Z acid pair no Mg$^{2+}$ binding was observed (72, 73). Thus, it would appear that both the -z and +z residues are important factors which contribute to cation selectivity. In conclusion, EF-hands utilize many factors to tune cation binding affinity.
CHAPTER 2

EXPERIMENTAL PROCEDURES

2.1 Proteins, peptides and materials

2'-Methylantraniloyl derivative of cyclic GMP (Mant-cGMP), 5,5'-
Br₂BAPTA (BAPTA), Chelex-100, Quin-2, mag-fura-2, fluo-3 and TNP-ATP were
purchased from Molecular Probes (Eugene, OR); TNS, EDTA and EGTA were
purchased from Sigma (St. Louis, MO) and hydroxylapatite was purchased from
Bio-Rad (Hercules, CA). RS-20, smooth muscle myosin light chain kinase CaM-
binding peptide (ARRKWQKTGHAVRAIGRLSS), was purchased from American
Peptide Co. (Sunnyvale, CA). All other chemicals were of analytical grade.

Site-directed mutagenesis and production of recombinant rat DNA-encoded
CaMs were done using previously described procedures (63). CaM was purified
from bovine brain and wheat germ as described previously by Kasturi et al. (74).
CaM concentrations were determined by extinction coefficient at 280 nm, with molar
absorbtivities for CaM mutants with and without F19W of 7200 M⁻¹s⁻¹ and
3140 M⁻¹cm⁻¹, respectively. Wheat germ CaM and skeletal TnC were fluorescently

35
labeled with 2-(4'-maleimidoanilino)-naphthalene-6-sulfonic acid (MIANS) (74) and
dansylaziridine (DANZ) (75), respectively. MLCK was purified to electrophoretic
homogeneity from chicken gizzard smooth muscle as described by Ngai et al. (76).
SR vesicles were prepared from the longissimus dorsi and white leg muscles of
rabbits by the method of Eletr and Inesi (77).

2.2 Methods

Kinetic measurements were performed by mixing an equal volume (50 ml) of
each reagent together in an Applied Photophysics Ltd. (Leatherhead, UK) model
SF.17 MV stopped-flow instrument, which has a dead time of 1.6 ms. The samples
were excited using a 150-watt Xenon arc source at the specified wavelength.
Fluorescence emission was monitored through the specified interference filters. The
curve fitting program (by P. J. King, Applied Photophysics Ltd.) uses the non-linear
Levenberg-Marquardt algorithm. The calibration of the change in quin-2
fluorescence into moles of Ca\(^{2+}\) dissociating from the Ca\(^{2+}\)-CaM-RS-20 complex was
previously described by Johnson et al. (43). All static fluorescence measurements
were performed on a Perkin-Elmer LS5 Spectrofluorimeter at room temperature.
Calcium titrations were performed using an EGTA buffered solution at pH 7.0 and
the [Ca\(^{2+}\)]\(_{free}\) was calculated as described by Robertson and Potter (78). Ca\(^{2+}\) binding
measurements using BAPTA were carried out by adding aliquots of Ca\(^{2+}\) to a
solution of 30 \(\mu\)M CaM or CaM mutant in the presence of 30 \(\mu\)M BAPTA. Ca\(^{2+}\)
binding to BAPTA was monitored by following its absorption at 262 nm and the
data were fit using a computer program developed and graciously provided by Dr.
Sara Linse, University of Lund, Lund Sweden (42). All proteins and buffers were
chelexed by incubation with chelex resin before Ca\(^{2+}\) binding studies were
performed. All the BAPTA titrations were done on a Beckman DU
spectrophotometer. The assays of Ca\(^{2+}\)-dependent activation of PDE by CaM were
performed as described previously by Johnson et al. (79).

Computer simulations were performed using KSIM version 1.1 (N.C. Millar,
UCLA School of Medicine, Los Angeles, [80]) which solved a set of differential
equations numerically using the Runge-Kutta method. All reactions were considered
to be bimolecular, with kinetic parameters (association and dissociation rates)
corresponding to those of the experimental data as listed in the appropriate figure
legends. The initial concentrations of the reagents in the simulations were set at the
steady state values corresponding to the contents of the two stopped-flow syringes
immediately after mixing (time = 0).
CHAPTER 3

CHARACTERIZING THE RESPONSE OF CALCIUM SIGNAL
TRANSDUCERS TO GENERATED CALCIUM TRANSIENTS

3.1 Introduction

Ca\(^{2+}\) is a ubiquitous second messenger responsible for regulating a large variety of cellular processes (81). In cells, [Ca\(^{2+}\)] rises in a transient fashion, allowing for the activation and subsequent inactivation of various Ca\(^{2+}\) binding-proteins and Ca\(^{2+}\)-regulated enzymes. The Ca\(^{2+}\) exchange rates of Ca\(^{2+}\)-binding proteins are variable (for review 9) allowing them to be "tuned" so that they are activated to a specific extent and for a particular duration by cellular Ca\(^{2+}\) transients. Alterations in the profile of Ca\(^{2+}\) transients or in the kinetics of Ca\(^{2+}\) exchange with Ca\(^{2+}\)-binding proteins can produce dramatic changes in Ca\(^{2+}\)-dependent physiological responses.

Fluorescent Ca\(^{2+}\) indicators, such as fluo-3 and mag-fura-2 (MF2), allow the time course of Ca\(^{2+}\) transients to be followed within living cells. The half widths (hws) of Ca\(^{2+}\) transients vary greatly from ~1 ms in neurons (82) to ~15 ms in skeletal muscle (83) and up to minutes in smooth muscle (84). Cytosolic [Ca\(^{2+}\)]
typically rises in cells when hormonal or neuronal stimulation triggers the entry of extracellular Ca$^{2+}$ and/or the release of Ca$^{2+}$ from internal stores (endoplasmic or sarcoplasmic reticulum (ER, SR)) (4, 85). Cytosolic [Ca$^{2+}$] decreases as Ca$^{2+}$-ATPases pump Ca$^{2+}$ into the ER or SR or extrude Ca$^{2+}$ across the plasma membrane. When Ca$^{2+}$ uptake mechanisms fail, Ca$^{2+}$ transients are prolonged and this can lead to cell necrosis and cell death (2). A transient rise in intracellular [Ca$^{2+}$] plays a fundamental role in activating various Ca$^{2+}$-binding proteins and Ca$^{2+}$-regulated enzymes which are essential for cardiac, skeletal and smooth muscle contraction, neurotransmission, energy metabolism, cell cycling and cell growth (1).

Nature has developed a variety of mechanisms to modify both the amplitude and duration of the Ca$^{2+}$ transient in order to evoke specific cellular responses. For example, β-adrenergic agonists increase the amplitude and shorten the duration of the Ca$^{2+}$ transient in cardiac ventricular muscle causing increased contractility and faster relaxation (86). Thus, the amplitude and duration of the Ca$^{2+}$ transient can dictate the extent and longevity of Ca$^{2+}$ binding to proteins and subsequent enzyme activation. It has also been shown in B lymphocytes that Ca$^{2+}$ transients of different amplitude and duration control the differential activation of specific transcriptional regulators (7). These studies imply that the tuning of Ca$^{2+}$ binding to proteins is also an important mechanism to alter cellular responses to changes in [Ca$^{2+}$].

Another example of this tuning mechanism is observed in skeletal muscle. During a twitch, Ca$^{2+}$ rapidly binds to the N-terminal regulatory sites of troponin C (TnC) to
elicit a contraction, while Ca\(^{2+}\) slowly exchanges with the Ca\(^{2+}\) buffering protein parvalbumin to aid relaxation (87). Thus, modifications of both the Ca\(^{2+}\) transient and Ca\(^{2+}\) exchange properties of Ca\(^{2+}\)-binding proteins can produce alterations in cell function.

Naturally occurring Ca\(^{2+}\) transients can be modified by varying electrical stimulation parameters (8), by drugs that inhibit Ca\(^{2+}\) resequestration into cellular compartments (88, 89) or by addition of extracellular (88) or intracellular (8, 90) Ca\(^{2+}\) chelators such as EGTA. These modifications have helped to determine the role Ca\(^{2+}\) plays in physiological systems. For example, in living skeletal muscle at 10\(^\circ\)C, intracellular EGTA caused a 2-fold decrease in the hw of twitch-induced Ca\(^{2+}\) transients, while the SR Ca\(^{2+}\)-ATPase inhibitor 2,5-di-(tert-butyl)-1,4-benzohydroquinone (TBQ) caused a 2-fold increase in the hw of the Ca\(^{2+}\) transient (8). As the duration of the Ca\(^{2+}\) transient was increased from ~40 ms to 130 ms, there was a linear increase in tension development. Thus, the duration of the Ca\(^{2+}\) transient in skeletal muscle is a primary determinant of force.

In these studies we show that when Ca\(^{2+}\) chelators with slow Ca\(^{2+}\) on-rates (EGTA and Mg\(^{2+}\)-EDTA) are rapidly mixed with Ca\(^{2+}\), the [Ca\(^{2+}\)] initially rises and then falls as these chelators bind Ca\(^{2+}\). This allowed us to generate Ca\(^{2+}\) transients of various amplitudes and durations in a stopped-flow-apparatus and to determine the response of Ca\(^{2+}\) indicators (flu-3 and MF2), Ca\(^{2+}\)-binding proteins (calmodulin (CaM) and TnC), and Ca\(^{2+}\)-dependent enzymes (myosin light chain kinase (MLCK) and SR Ca\(^{2+}\)-ATPase) to these Ca\(^{2+}\) transients. Previously investigators determined
Ca\(^{2+}\) on-rates by rapidly mixing Ca\(^{2+}\) with an apo Ca\(^{2+}\)-binding protein and determined Ca\(^{2+}\) off rates by mixing a Ca\(^{2+}\) chelator with a Ca\(^{2+}\)-saturated Ca\(^{2+}\)-binding protein. Our new approach allows us to follow the consequences of both Ca\(^{2+}\) binding and Ca\(^{2+}\) removal including: Ca\(^{2+}\)-induced conformational changes; Ca\(^{2+}\)-induced protein/peptide and protein/protein interactions; and enzyme activation and inactivation, in response to Ca\(^{2+}\) transients of various amplitude and duration. This has enabled us to define how these Ca\(^{2+}\)-binding proteins and Ca\(^{2+}\)-dependent enzymes are tuned to receive and transduce the Ca\(^{2+}\) signal into cellular actions. This approach should further our understanding of how cellular Ca\(^{2+}\) transients regulate Ca\(^{2+}\)-dependent processes.

3.2 Results

3.2.1 Response of a rapid and a slow Ca\(^{2+}\) indicator to a 1.2 ms hw artificial Ca\(^{2+}\) transient

Since EGTA has a slow Ca\(^{2+}\) on-rate, when Ca\(^{2+}\) is rapidly mixed with EGTA, free [Ca\(^{2+}\)] initially rises and then falls as it is chelated by EGTA. The slow Ca\(^{2+}\) chelation by EGTA thereby allows the production of artificial Ca\(^{2+}\) transients (ACTs) in a stopped-flow apparatus. The KSIM computer program (80) was used to simulate the Ca\(^{2+}\) transient which would be produced when 100 \(\mu\)M Ca\(^{2+}\) was instantaneously mixed with 1 mM EGTA. This simulation (Figure 3.1, Ca trace; also see table 3.1 for rate constants) shows that [Ca\(^{2+}\)]free initially set at 100 \(\mu\)M, would fall at a rate of 600/s producing an ACT with a 1.2 ms hw. This ACT can be
produced and visualized by mixing 200 μM Ca²⁺ with the rapid Ca²⁺ indicator MF2 in the presence of 1 mM EGTA, in a stopped-flow apparatus at 10°C (Figure 3.1, Mag-Fura-2 trace). Initially, Ca²⁺ occupied ~80% of the MF2 causing an increase in its fluorescence during the mixing time of the instrument. As the Ca²⁺ was subsequently chelated by EGTA, MF2 fluorescence decreased at a rate of ~600/s which corresponded to the simulated rate of fall in [Ca²⁺] for this ACT. Thus, the ACT produced when Ca²⁺ is mixed with EGTA can be visualized using MF2 fluorescence. When the higher affinity Ca²⁺ indicator, Fluo-3, was subjected to the same ACT (Figure 3.1, Fluo-3 trace), Ca²⁺ initially saturated the indicator causing an increase in its fluorescence during the mixing time of the instrument. As Ca²⁺ was subsequently chelated by EGTA, fluo-3 fluorescence decreased ~6 times more slowly (100/s) than the MF2 fluorescence and the simulated rate of fall in [Ca²⁺]. Thus, the rapid Ca²⁺ indicator MF2 accurately reported this ACT while the slower Ca²⁺ indicator fluo-3 responded to the same ACT with a large kinetic delay.
Figure 3.1: Response of the Ca^{2+} indicators mag-fura-2 and fluo-3 to a 1.2 ms half width artificial Ca^{2+} transient.

The traces show the time courses of decrease in [Ca^{2+}]_{free} (Ca^{2+} trace), mag-fura-2 fluorescence (Mag-Fura-2 trace, inverted for comparison) and fluo-3 fluorescence (Fluo-3 trace) during a 1.2 ms hw artificial Ca^{2+} transient. 200 μM Ca^{2+} in 10 mM Mops, 90 mM KCl, pH 7.0 was rapidly mixed with an equal volume of 1 μM of the appropriate Ca^{2+} indicator + 1 mM EGTA, in the same buffer, at 10°C. The simulation of fall in [Ca^{2+}]_{free} of this artificial Ca^{2+} transient was modeled using the program KSIM (80). The initial [Ca^{2+}] was set at 100 μM and the initial [EGTA] at 500 μM. The kinetic parameters in the simulation for the Ca^{2+} on and off-rates from EGTA of 0.55/s and 1.3x10^6 M^{-1} s^{-1} at 10°C, respectively were used. The half width of the artificial Ca^{2+} transient was measured as the time required for a 50% decrease in [Ca^{2+}]_{free}. Control experiments, in which Ca^{2+} + mag-fura-2 or fluo-3 were mixed with buffer + Ca^{2+} were flat and indicated that ~80% of mag-fura-2 and >95% of fluo-3 were transiently occupied with Ca^{2+} during this artificial Ca^{2+} transient. Mag-fura-2 fluorescence was monitored through a 510 nm broad band pass filter (Oriel, Stanford, CT) with excitation at 380 nm. Fluo-3 fluorescence was monitored through a 530 nm narrow band pass filter (Oriel, Stanford, CT) with excitation at 490 nm. Each fluorescence trace represents an average of 5 traces fit with a single exponential equation (variance < 2x10^{-4}).
<table>
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<tr>
<th>Complexes</th>
<th>Kon On Rate (M⁻¹s⁻¹)</th>
<th>Koff Off Rate (s)</th>
<th>Kd Affinity (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGTA + Ca²⁺</td>
<td>1.3 x10⁶</td>
<td>0.55</td>
<td>423 x10⁻⁹</td>
</tr>
<tr>
<td>EDTA + Ca²⁺</td>
<td>2.2 x10⁷</td>
<td>0.7</td>
<td>32 x10⁻⁹</td>
</tr>
<tr>
<td>EDTA + Mg²⁺</td>
<td>8.75 x10⁵</td>
<td>2.8</td>
<td>32 x10⁻⁷</td>
</tr>
<tr>
<td>MF2 + Ca²⁺</td>
<td>2 x10⁸</td>
<td>4000</td>
<td>2 x10⁻⁵</td>
</tr>
<tr>
<td>Fluo-3 + Ca²⁺</td>
<td>6.4 x10⁶</td>
<td>170</td>
<td>315 x10⁻⁹</td>
</tr>
<tr>
<td>TnC N-Terminal + Ca²⁺</td>
<td>8 x 10⁷</td>
<td>160</td>
<td>2 x 10⁻⁶</td>
</tr>
<tr>
<td>CaM C-Terminal + Ca²⁺</td>
<td>2.3 x10⁶</td>
<td>2.4</td>
<td>1 x10⁻⁶</td>
</tr>
<tr>
<td>CaM N-Terminal + Ca²⁺</td>
<td>1.6 x10⁸</td>
<td>405</td>
<td>25 x10⁻⁷</td>
</tr>
<tr>
<td>CaM N-Terminal-Ca²⁺ + MLCK</td>
<td>6.7 x10⁷</td>
<td>0.031</td>
<td>5 x10⁻¹⁰</td>
</tr>
</tbody>
</table>

Table 3.1: Comparison of the association rates, dissociation rates and affinities for the Ca²⁺ indicators and proteins exposed to ACTs calculated or measured for 10°C.
3.2.2 Effect of [EGTA] on ACT Duration

Figure 3.2 shows the time courses of several ACTs when Ca\(^{2+}\) was rapidly mixed with MF2 in the presence of increasing [EGTA]. When 200 \(\mu\text{M} \text{Ca}^{2+}\) was mixed with 1\(\mu\text{M}\) MF2 in the presence of 0.5 mM EGTA (Figure 3.2, 0.5 mM EGTA trace), \(\text{Ca}^{2+}\) occupied \(\sim80\%\) of the MF2 causing its fluorescence to increase during the mixing time of the instrument. As \(\text{Ca}^{2+}\) was subsequently chelated by EGTA, MF2 fluorescence decreased at a rate of \(\sim315/s\) back to its \(\text{Ca}^{2+}\)-free fluorescence level. Under these conditions, an ACT with an amplitude of 100 \(\mu\text{M} \text{Ca}^{2+}\) and a hw of 2.2 ms (determined from the extrapolated single exponential fit to the data) was produced. As [EGTA] was successively increased from 0.5 to 0.75, 1 and 2 mM (Figure 3.2), ACTs of 1.6, 1.2 and 0.6 ms hws were produced. The rate of fall in \([\text{Ca}^{2+}]\) in these ACTs increased linearly with increasing [EGTA], as expected for a second order reaction. At higher [EGTA], more of the \(\text{Ca}^{2+}\) was chelated during the \(\sim1.6\) ms mixing time of the instrument resulting in less of the decrease in MF2 fluorescence being observed. Thus, ACTs which vary in duration from 0.6 to 2.2 ms hws can be generated using various concentrations of the slow \(\text{Ca}^{2+}\) chelator EGTA. When 200 \(\mu\text{M} \text{Ca}^{2+}\) was mixed with 1\(\mu\text{M}\) MF2 in the presence of 500 \(\mu\text{M}\) EGTA, at 10, 20 and 30\(^\circ\text{C}\), the hw of the ACT decreased from 2.2 ms to 1.1 ms to 0.6 ms, respectively (data not shown). Thus, the duration of an ACT generated with EGTA exhibits a Q\(_{10}\) of \(\sim2.0\).
Figure 3.2: Effect of [EGTA] on the half width of artificial Ca\(^{2+}\) transients.

Mag-fura-2 fluorescence (traces inverted) was used to follow the time course of the artificial Ca\(^{2+}\) transients which were produced when 200 μM Ca\(^{2+}\) was rapidly mixed with an equal volume of 1μ M mag-fura-2 in the presence of 0.5, 0.75, 1 or 2 mM EGTA. Experiments were conducted at 10°C using the buffers described in the legend of Figure 3.1. Control experiments, in which Ca\(^{2+}\) + mag-fura-2 was mixed with buffer + Ca\(^{2+}\), were flat and indicated that ~80% of the mag-fura-2 was transiently occupied with Ca\(^{2+}\) during each artificial Ca\(^{2+}\) transient. Mag-fura-2 fluorescence was monitored as described in the legend of Figure 3.1. Each trace represents an average of 5 traces fit with a single exponential equation (variance < 3x10\(^{-4}\)).
3.2.3 Generation of Longer Duration ACTs Using EDTA

When the Ca$^{2+}$ chelator EDTA is bound to Mg$^{2+}$, Ca$^{2+}$ cannot bind until Mg$^{2+}$ dissociates. Since Mg$^{2+}$ dissociates from EDTA at ~3/s at 10°C, Mg$^{2+}$-EDTA is a slow Ca$^{2+}$ chelator. Therefore, when Ca$^{2+}$ is mixed with Mg$^{2+}$-EDTA, [Ca$^{2+}$]$_{\text{free}}$ initially rises and then falls as Ca$^{2+}$ displaces Mg$^{2+}$ and binds to EDTA. The very slow Ca$^{2+}$ chelation by Mg$^{2+}$-EDTA allows the production of ACTs of longer duration than those produced with EGTA. Figure 3.3A shows the simulated time course of the fall in [Ca$^{2+}$]$_{\text{free}}$ when 10 μM Ca$^{2+}$ was rapidly mixed with 100 μM EDTA in the presence of increasing [Mg$^{2+}$] (see table 3.1 for rate constants). This simulation shows that in the absence of Mg$^{2+}$ (Figure 3.3A, No Mg trace) an ACT with an amplitude of 10 μM Ca$^{2+}$ and an ~0.3 ms hw was produced. As [Mg$^{2+}$] was successively increased to 0.25, 0.5 and 1 mM, the hw of the ACT progressively increased to 5, 23 and 50 ms, respectively (Figure 3.3A). Thus, as [Mg$^{2+}$] was increased from 0 to 1 mM, the rate of fall in the Ca$^{2+}$ transient was reduced from ~2,300/s to ~14/s, producing an ~165-fold increase in ACT duration.

Fluo-3 was used to visualize the ACT produced with Mg$^{2+}$-EDTA since fluo-3 fluorescence is not affected by Mg$^{2+}$. Figure 3.3B shows fluo-3 fluorescence as a function of time when fluo-3 is subjected to ACTs generated with EDTA in the presence of increasing [Mg$^{2+}$]. When 20 μM Ca$^{2+}$ was mixed with 1 μM Fluo-3 in the presence of 200 μM EDTA, Ca$^{2+}$ initially occupied ~100% of the fluo-3 causing an increase in its fluorescence during the mixing time of the instrument. As Ca$^{2+}$ was
subsequently chelated by EDTA, fluo-3 fluorescence decayed at a rate of \(\sim 200/s\) (3 ms hw) which was \(\sim 10\) times slower than the simulated rate of fall in \([\text{Ca}^{2+}]\) at \(\sim 2,000/s\) (0.3 ms hw) in this ACT. As \([\text{Mg}^{2+}]\) was increased to 0.25, 0.5 and 1 mM, \(\text{Ca}^{2+}\) initially saturated fluo-3 causing an increase in its fluorescence during the mixing time of the instrument. Upon increasing \([\text{Mg}^{2+}]\) to these reactions, Fluo-3 is saturated with \(\text{Ca}^{2+}\) for increasing periods of time before \(\text{Ca}^{2+}\) can displace \(\text{Mg}^{2+}\) from EDTA and be effectively removed from Fluo-3. Once this occurred, fluo-3 fluorescence (Figure 3.3B) decreased with hw of \(\sim 30\) (23/s), 50 (14/s) and 116 ms (6/s) for 0.25, 0.5 and 1 mM \(\text{Mg}^{2+}\), respectively. As the hw of the simulated ACT increased from 0.3 ms to 5, 23 and 50 ms, fluo-3 reported the decrease in \([\text{Ca}^{2+}]\) more accurately with decreasing kinetic delays from \(\sim 10\) to 2-fold. Due to the competitive binding of \(\text{Ca}^{2+}\) and \(\text{Mg}^{2+}\) to EDTA, as the \([\text{Mg}^{2+}]\) was increased EDTA lost some of its capacity to remove \(\text{Ca}^{2+}\) from fluo-3 and this is reflected by the fact that fluo-3 fluorescence does not return to its \(\text{Ca}^{2+}\)-free state at higher \([\text{Mg}^{2+}]\). These studies show that at a fixed \([\text{EDTA}]\), increasing \([\text{Mg}^{2+}]\) produces more of the slow \(\text{Ca}^{2+}\) chelator \(\text{Mg}^{2+}\)-EDTA which allows for the creation of ACTs with longer duration (from 0.3 to 50 ms hw).
Figure 3.3: Effect of [Mg$^{2+}$] on the half width of artificial Ca$^{2+}$ transients created with EDTA.

A. The traces show computer simulations (using KSIM) of the artificial Ca$^{2+}$ transients produced when 10 μM Ca$^{2+}$ was instantaneously mixed with 100 μM EDTA in the absence of Mg$^{2+}$ (No Mg trace), or in the presence of 0.25, 0.5 or 1 mM Mg$^{2+}$. At time = 0, the initial [Mg$^{2+}$], [EDTA] and [Mg$^{2+}$-EDTA] were set at the steady-state values for the specific [Mg$^{2+}$] used, and then rapidly mixed with 10 μM Ca$^{2+}$. Ca$^{2+}$ on and off-rates (2.2x10$^7$ M$^{-1}$ s$^{-1}$ and 0.7/s, respectively) and Mg$^{2+}$ on and off-rates (8.75x10$^5$ M$^{-1}$ s$^{-1}$ and 2.8/s, respectively) from EDTA at 10°C were used in these simulations. The half widths of the artificial Ca$^{2+}$ transients were measured as the time required for a 50% decrease in [Ca$^{2+}$]$_{free}$. B. The traces show the time course of the decrease in fluo-3 fluorescence that occurs when 20 μM Ca$^{2+}$ was mixed with an equal volume of 1 μM fluo-3 + 200 μM EDTA, in the absence of Mg$^{2+}$ or in the presence of 0.25, 0.5 or 1 mM Mg$^{2+}$, at 10°C. The control experiment, in which Ca$^{2+}$ + fluo-3 were mixed with buffer + Ca$^{2+}$, was flat and indicated that >95% of the fluo-3 was transiently occupied with Ca$^{2+}$ during each artificial Ca$^{2+}$ transient. Buffers and measurement of fluo-3 fluorescence were as described in the legend of Figure 3.1. Each trace represents an average of 5 traces fit with a single exponential equation (variance < 9x10$^{-5}$) after the flat lag phase which represents the Ca$^{2+}$-saturated state.
Figure 3.3: Effect of $[\text{Mg}^{2+}]$ on the half width of artificial $\text{Ca}^{2+}$ transient created with EDTA.
3.2.4 Using ACTs to Estimate the Ca\(^{2+}\) On Rate to the N-terminal Sites of TnC

TnC-danz is a fluorescently labeled skeletal muscle TnC which undergoes a large increase in fluorescence when its N-terminal regulatory sites bind Ca\(^{2+}\) (91). TnC-danz has a similar Ca\(^{2+}\) affinity (half-maximal at pCa 5.8) and Ca\(^{2+}\) dissociation rate (~350/s, at 22°C) as wild-type TnC (92). Figure 3.4 uses the changes in TnC-danz fluorescence to show the time course of Ca\(^{2+}\) binding and dissociation from the N-terminal Ca\(^{2+}\)-specific sites of TnC during ACTs of increasing duration. When 200 µM Ca\(^{2+}\) was mixed with 2 µM TnC-danz in the presence of 0.75 mM EGTA (Figure 3.4, 0.75 mM EGTA trace) a 1.6 ms hw ACT was generated and Ca\(^{2+}\) bound and transiently occupied ~100% of the Ca\(^{2+}\)-specific regulatory sites of TnC-danz during the mixing time of the instrument. As Ca\(^{2+}\) was subsequently chelated by EGTA and removed from TnC-danz, the fluorescence decayed back to its Ca\(^{2+}\)-free fluorescence level at a rate of ~95/s. As [EGTA] was successively increased from 0.75 mM to 5, and 10 mM EGTA (Figure 3.4) ACTs of 0.21 and 0.1 ms hw were created and Ca\(^{2+}\) transiently occupied ~75 and 50% of the N-terminal regulatory sites of TnC-danz, respectively. Furthermore, as [EGTA] was increased from 0.75 to 5 and 10 mM EGTA, Ca\(^{2+}\) was chelated and removed from TnC-danz more quickly at rates of 145 and 160/s, respectively. Since the percent occupancy of Ca\(^{2+}\) bound to the N-terminal of TnC-danz during an ACT of a given duration is directly related to the rate of Ca\(^{2+}\) binding to those sites, we were able to estimate the Ca\(^{2+}\) on-rate to the regulatory sites of TnC-danz. We used a computer simulation which fixed the Ca\(^{2+}\) off-rate from TnC-danz at 140/s (Ca\(^{2+}\) off-rate at 10°C, see table 3.1)
and let the Ca$^{2+}$ on-rate to TnC-danz vary from $1.0 \times 10^6$ to $5.0 \times 10^8$ M$^{-1}$ s$^{-1}$ until the modeled transient occupancy and Ca$^{2+}$ dissociation rates approximated the experimental data. The modeling (see Appendix, figure 5.1) predicts that we should observe the percent occupancies obtained experimentally if the Ca$^{2+}$ on-rate to the Ca$^{2+}$-specific regulatory sites of TnC-danz is $\sim 8.0 \pm 0.1 \times 10^7$ M$^{-1}$ s$^{-1}$, at 10°C. Thus, by exposing a Ca$^{2+}$-binding protein to ACTs of different duration and examining the percent occupancy as a function of Ca$^{2+}$ transient duration, we can determine the rate of Ca$^{2+}$ binding to that protein.
Figure 3.4: Ca\(^{2+}\) occupancy of the N-terminal Ca\(^{2+}\) specific regulatory sites of troponin C during artificial Ca\(^{2+}\) transients of different duration.

The traces show the time courses of decrease in TnC-danz fluorescence when 200 \(\mu\)M Ca\(^{2+}\) was mixed with an equal volume of 2 \(\mu\)M TnC-danz in the presence of 0.75, 5, or 10 mM EGTA, at 10\(^\circ\)C. The control experiment (No EGTA trace), in which TnC-danz was mixed with buffer + Ca\(^{2+}\), was flat and considered to be 100\% transient occupancy of TnC-danz. TnC-danz fluorescence was monitored through a 510 nm broad band pass filter (Oriel, Stanford, CT) with excitation at 340 nm. Buffers are those described in the legend of Figure 3.1. Each trace represents an average of 5 traces fit with a single exponential equation (variance <2x10\(^{-4}\)).
3.2.5 Quin-2 and EGTA Dissociation of Ca\(^{2+}\) and RS-20 from the Ca\(^{2+}\)-CaM-RS-20 Complex.

Ca\(^{2+}\) binding to the N- or C-terminal of CaM exposes hydrophobic pockets in both domains that allow CaM to bind target peptides and proteins (for review 93). The binding of target peptides to CaM increases CaM’s N- and C-terminal Ca\(^{2+}\) affinities ~200 and 20-fold respectively, by primarily slowing the Ca\(^{2+}\) dissociation rates (42). Figure 3.5 (Quin-2 trace) shows the time course of increase in Quin-2 fluorescence that occurred as 2 moles of Ca\(^{2+}\) were removed from both the N-terminal (2/s) and C-terminal (0.1/s) sites of CaM bound to RS-20. Figure 3.5 (Trp trace) shows the time course of the EGTA-induced increase in RS-20 Trp fluorescence that occurred as RS-20 dissociated from CaM’s N-terminal (2/s) and C-terminal (0.1/s). Clearly, RS-20 dissociates from the N- and C-terminals of CaM at the rates at which Ca\(^{2+}\) dissociates from the N- and C-terminals of CaM in the Ca\(^{2+}\)-CaM-RS-20 complex.
Figure 3.5: The time course of Ca\(^{2+}\) and of RS-20 dissociation from the Ca\(^{2+}\)-CaM-RS-20 complex.

The Quin-2 trace shows the increase in Quin-2 fluorescence that occurs upon Ca\(^{2+}\) dissociation from the N- and C-terminals of CaM in the Ca\(^{2+}\)-CaM-RS-20 complex. 60 µM Ca\(^{2+}\) + 4 µM CaM + 8 µM RS-20 in 20 mM Hepes, pH 7.0, was rapidly mixed with an equal volume of 200 µM quin-2 in the same buffer at 10°C. Quin-2 fluorescence was monitored with a 510 nm broad band pass filter (Oriel, Stanford, CT) with excitation at 330 nm. The trace represents an average of 5 traces fit with a double exponential equation (variance <8x10\(^{-5}\)). The Trp trace shows the increase in RS-20 tryptophan fluorescence that occurs when 75 µM Ca\(^{2+}\) + 4 µM CaM + 8 µM RS-20 in 20 mM Hepes, pH 7.0, was rapidly mixed with an equal volume of 20 mM EGTA in the same buffer at 10°C. RS-20 tryptophan fluorescence was monitored through a UV-transmitting black glass filter (UG1 (Oriel, Stanford, CT)) with excitation at 275 nm. The trace is an average of 5 traces fit with a double exponential equation (variance <5x10\(^{-5}\)).
3.2.6 Calmodulin Binding to RS-20 Following a Rapid ACT

The fact that RS-20 Trp fluorescence decreases when it binds to both the N- and C-terminals of CaM allowed us to determine if the N- or C-terminal of CaM could bind to RS-20 during a rapid ACT. Figure 3.6 shows the EGTA-induced increase in RS-20 Trp fluorescence when EGTA (20 mM) is rapidly mixed with CaM (4 µM) and RS-20 (8 µM) in the presence of 75 µM Ca²⁺. There is a biphasic increase in RS-20 Trp fluorescence; 50% occurs at 2/s as Ca²⁺ dissociates from the N-terminal (Figure 3.6, trace A.(N-EGTA)) and 50% occurs at 0.1/s as Ca²⁺ dissociates from the C-terminal (Figure 3.6, trace A.(C-EGTA)) of the CaM-RS-20 complex.

Using these changes in RS-20 fluorescence we were able to determine if either the N- and/or C-terminal of CaM could transiently associate with RS-20 during a rapid ACT. Figure 3.6 (trace B) shows the change in RS-20 fluorescence that was observed when 75 µM Ca²⁺ was mixed with CaM (4 µM), in the presence of RS-20 (8 µM) and 1 mM EGTA. Following this 1.1 ms hw ACT, Ca²⁺ binds to CaM and CaM then binds to RS-20 producing a decrease in RS-20 Trp fluorescence during the mixing time of the apparatus. This decrease in fluorescence was followed by a biphasic increase in RS-20 Trp fluorescence. Most (~90%) of this increase in Trp fluorescence occurred as RS-20 dissociated from the N-terminal (Figure 3.6, trace B.(N-Transient)) of CaM at 2/s and the remaining ~10% occurred as RS-20 dissociated from the C-terminal (Figure 3.6, trace B. (C-Transient)) of CaM at 0.1/s.
Comparing the amplitudes of the increase in RS-20 fluorescence that occurred in the ACT experiment (Figure 3.6, trace B) to the amplitudes of the increase in the EGTA experiment (Figure 3.6, trace A), suggests that 90% of the N-terminal, but only 10% of the C-terminal sites of CaM are able to transiently bind to RS-20 during this 1.1 ms hw ACT. Thus, during a rapid ACT only the N-terminal of CaM has a fast enough Ca$^{2+}$ on-rate and conformational change to allow it to transiently associate with this target peptide (see table 3.1).
Figure 3.6: The time course of the decrease in Ca\(^{2+}\)-CaM-RS-20 Trp fluorescence upon Ca\(^{2+}\) chelation by EGTA and after a 1.1 ms half width artificial Ca\(^{2+}\) transient.

The time course of the increase in RS-20 tryptophan fluorescence is shown as EGTA dissociates the Ca\(^{2+}\)-CaM-RS-20 complex (trace A) or when the complex has been transiently formed by a rapid ACT (trace B). The inset shows these two reactions over shorter times with the two traces staggered for comparison. The EGTA-induced increase in RS-20 tryptophan fluorescence (trace labeled A. N-EGTA and A. C-EGTA) was accomplished by mixing 75 \(\mu\)M Ca\(^{2+}\) + 4 \(\mu\)M CaM + 8 \(\mu\)M RS-20 with an equal volume of 20 mM EGTA at 10\(^\circ\)C. The transient occupancy of CaM with Ca\(^{2+}\) and RS-20 (trace labeled B. (N-Transient) and B. (C-Transient)) was accomplished by mixing 75 \(\mu\)M Ca\(^{2+}\) with an equal volume of 4 \(\mu\)M CaM + 8 \(\mu\)M RS-20 + 1 mM EGTA at 10\(^\circ\)C. Buffers and measurements of tryptophan fluorescence are described in the legend of Figure 3.5. All traces are an average of 5 traces fit with a double exponential equation (variance <5x10\(^{-5}\)).
3.2.7 CaM Binding to MLCK Following ACTs of Various Duration

Wheat CaM-MIANS exhibits a large fluorescence increase which is specific for its binding to target proteins including MLCK (74). This fluorescent CaM allowed us to determine if CaM could bind MLCK after exposure to ACTs of varying duration. Figure 3.7A uses MF2 fluorescence to visualize the 0.6 ms hw ACT which is produced when 200 μM Ca\(^{2+}\) is mixed with 2 mM EGTA (Mag-Fura-2 trace). \([\text{Ca}^{2+}]_{\text{free}}\) increases during the mixing time of the instrument and decays at a rate of 1200/s. When CaM-MIANS, in the presence of MLCK and EGTA, was exposed to this ACT, CaM-MIANS fluorescence increased at a rate of ~90/s (Figure 3.7A, CaM\(_{\text{MIANS}}\) + MLCK trace). There was an ~25 ms delay between peak \([\text{Ca}^{2+}]\) and peak CaM-MIANS binding to MLCK. In fact, the \([\text{Ca}^{2+}]_{\text{free}}\) had fallen essentially back to baseline before ~20 to 30% of the increase in CaM-MIANS fluorescence (Ca\(^{2+}\)-CaM-MLCK complex formation) was detected. Since we are exposing CaM and MLCK to a Ca\(^{2+}\) transient, the CaM-MIANS-MLCK complex should dissociate after Ca\(^{2+}\) has been chelated by EGTA. Figure 3.7B shows the same trace (2 mM EGTA trace) over longer times (0 to 5 s). CaM-MIANS fluorescence increased at 90/s and then decreased at a rate of ~1/s. Thus, when subjected to a 0.6 ms hw ACT, CaM-MIANS is able to rapidly and transiently bind MLCK. The No EGTA trace (Figure 3.7B) shows the CaM-MIANS fluorescence when it is saturated with MLCK. Thus, a 0.6 ms hw ACT allows ~60% of CaM-MIANS to transiently complex with MLCK. Figure 3.7B also shows the transient binding of CaM-MIANS to MLCK following ACTs with hws of 0.2 and 1.1 ms. A
shorter, 0.2 ms hw, ACT (Figure 3.7B, 5 mM EGTA trace) resulted in ~35% of the CaM-MIANS transiently binding to MLCK, with similar kinetics as the 2 mM EGTA trace. A longer, 1.1 ms hw, ACT (Figure 3.7B, 1 mM EGTA trace) resulted in ~80% of the CaM-MIANS transiently binding to MLCK, again with similar kinetics. Thus, under the conditions used in these experiments a ~0.4 ms hw ACT is required for ~50% of the CaM to transiently complex with MLCK. This suggests that the N-terminal of CaM can bind to MLCK even during very rapid Ca^{2+} transients.
Figure 3.7: The binding of CaM-MIANS to MLCK during artificial Ca\(^{2+}\) transients of different duration.

A. The time course of increase in mag-fura-2 fluorescence (Mag-Fura-2 trace, inverted for comparison) and increase in CaM-MIANS fluorescence (CaM\(_{MIANS}\) + MLCK trace) produced by a 0.6 ms half width artificial Ca\(^{2+}\) transient. The transient binding of CaM-MIANS to MLCK was produced by rapidly mixing 200 \(\mu\)M Ca\(^{2+}\) with 200 nM CaM-MIANS + 400 nM MLCK + 1 mM EGTA. The Mag-Fura-2 trace was produced by rapidly mixing 200 \(\mu\)M Ca\(^{2+}\) with 1 \(\mu\)M mag-fura-2 + 1 mM EGTA. B. Shows the time course of the rise and fall in CaM-MIANS fluorescence obtained when 200 \(\mu\)M Ca\(^{2+}\) was rapidly mixed with 200 nM CaM-MIANS + 400 nM MLCK in the presence of 1, 2 or 5 mM EGTA. CaM-MIANS fluorescence was monitored using a 420-470 nm broad band pass filter (Oriel, Stanford, CT) with excitation at 320 nm while mag-fura-2 fluorescence was monitored as described in the legend of Figure 3.1. Experiments were conducted at 10°C using the buffers described in the legend of Figure 3.1. Control trace, in which no EGTA (No EGTA trace) was used, shows maximal association of CaM-MIANS with MLCK and is considered 100% binding. Each trace represents an average of 5 traces fit with a double exponential equation (CaM-MIANS fluorescence) or a single exponential equation (mag-fura-2 fluorescence) (variance <4x10\(^{-4}\)).
Figure 3.7: The binding of CaM-MIANS to MLCK during artificial Ca$^{2+}$ transients of different duration.
3.2.8 Transient Activation of the SR Ca\(^{2+}\)-ATPase by ACTs of Different Duration

Watanabe and Inesi (94) have previously shown that the Ca\(^{2+}\)-ATPase activity of SR vesicles can be followed by the fluorescent nucleotide 2',3'-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP). TNP-ATP undergoes a large fluorescence increase which is proportional to the formation of the phospho-enzyme intermediate (E1\(\sim\)P) when the SR Ca\(^{2+}\)-ATPase is activated by Ca\(^{2+}\) and Mg\(^{2+}\)-ATP (94, 95). This fluorescence decays as E1\(\sim\)P is inactivated by Ca\(^{2+}\) chelation or by ATP depletion. Figure 3.8A shows a 0.9 ms hw ACT produced when 200 \(\mu\)M Ca\(^{2+}\) was mixed with 600 \(\mu\)M EGTA as monitored by the change in MF2 fluorescence (Mag-Fura-2 trace). When vesicles containing the SR Ca\(^{2+}\)-ATPase were exposed to this ACT, TNP-ATP fluorescence increased at a rate of \(-83/s\) (Figure 3.8A, TNP-ATP trace). There was an \(-32\) ms delay between peak [Ca\(^{2+}\)] and peak activation of the SR Ca\(^{2+}\)-ATPase. In fact, the Ca\(^{2+}\) transient had fallen back to baseline before any significant activation of the Ca\(^{2+}\)-ATPase occurred. Since this enzyme was activated by a transient increase in Ca\(^{2+}\), it should inactivate after the Ca\(^{2+}\) transient subsides. Figure 3.8B shows the same trace (600 \(\mu\)M EGTA trace) over longer times (0 to 1 s). TNP-ATP fluorescence increases at 83/s and then decays back to the no Ca\(^{2+}\) state (2 mM EGTA trace) at a rate of 4/s. By comparing the Ca\(^{2+}\)-induced activation of the SR Ca\(^{2+}\)-ATPase obtained in the absence of EGTA and with saturating [Ca\(^{2+}\)] (data not shown), we determined that the SR Ca\(^{2+}\)-ATPase is transiently activated to \(-14\)% of its maximal activity by this 0.9 ms hw ACT. Figure 3.8B also shows that a
shorter duration, 0.7 ms hw, ACT (Figure 3.8B, 800 μM EGTA trace) resulted in lower (5%) transient activation of the SR Ca$^{2+}$-ATPase while a longer duration, 1.8 ms hw, ACT (Figure 3.8B, 400 μM EGTA trace) resulted in greater (22%) transient activation of the enzyme, all with similar kinetics. Thus, the SR Ca$^{2+}$-ATPase is able to bind Ca$^{2+}$ and become activated after a brief kinetic delay by very rapid ACTs.
Figure 3.8: Time course of activation of the SR Ca$^{2+}$-ATPase by artificial Ca$^{2+}$ transients of different duration.

A. The time course of increase in mag-fura-2 fluorescence (Mag-Fura-2 trace, inverted for comparison) and TNP-ATP fluorescence (TNP-ATP trace) by a 0.9 ms half width artificial Ca$^{2+}$ transient. The transient activation of the SR Ca$^{2+}$-ATPase was produced by rapidly mixing 200 μM Ca$^{2+}$ with 0.6 mg/ml SR protein in the presence of 10 μM TNP-ATP and 600 μM EGTA. The Mag-Fura-2 trace was produced by rapidly mixing 200 μM Ca$^{2+}$ with 1 μM mag-fura-2 in the presence of 1 mM ATP and 600 μM EGTA. TNP-ATP fluorescence was monitored using a 547 nm band pass filter (Oriel, Stanford, CT) with excitation at 405 nm. Mag-fura-2 fluorescence was monitored as described in the legend of Figure 3.1. Each trace represents an average of 8 traces fit with a single exponential equation (variance < 2x10^{-4}).

B. Shows the time course of the rise and fall in TNP-ATP fluorescence (SR Ca$^{2+}$-ATPase transient activation) obtained when the [EGTA] in the above reactions was decreased from 2 mM to 800 μM, 600 μM or 400 μM. The control experiment, in which 1.3 mM Ca$^{2+}$ was used to maximally activate the SR Ca$^{2+}$-ATPase (100%), remained activated for >5 min. Experiments were conducted in 20 mM Tris-Mal and 20% glycerol at 20°C. Each trace represents an average of 8 traces. Both the increase and decrease in TNP-ATP fluorescence was fit with a single exponential equation (variance < 2x10^{-4}).
Figure 3.8: Time course of activation of the SR Ca\(^{2+}\)-ATPase by artificial Ca\(^{2+}\) transients of different duration.
3.3 Discussion

Because protons must dissociate from EGTA before it can bind Ca\(^{2+}\) (96), EGTA has a slow Ca\(^{2+}\) on-rate (1 - 3 x 10\(^{6}\) M\(^{-1}\) s\(^{-1}\)) (43, 96), and can be considered a "slow" Ca\(^{2+}\) chelator. Thus, when EGTA is rapidly mixed with Ca\(^{2+}\) in a stopped-flow apparatus, [Ca\(^{2+}\)] rises before it is chelated and this produces an ACT. Since Ca\(^{2+}\) binding to EGTA is a second order reaction, as the [EGTA] is increased, faster ACTs are produced and these can be accurately visualized with the Ca\(^{2+}\) indicator MF2 and modeled by computer simulations. Using 200 \(\mu\)M Ca\(^{2+}\) and increasing [EGTA], ACTs with hws from 16 to 0.1 ms and 100 \(\mu\)M amplitude were created in a stopped-flow apparatus. The amplitude of the ACT could be increased or decreased by mixing more or less Ca\(^{2+}\) with a fixed [EGTA]. Using this method, ACTs with different amplitudes and similar hws can be created. However, if the [EGTA] becomes less than the [Ca\(^{2+}\)] by more than ~5-fold, the duration of the ACT increases as the chelating potential of EGTA is reduced. Furthermore, since the Ca\(^{2+}\) exchange rates of EGTA increase with temperature, we determined that the hw of an ACT generated with EGTA exhibits a Q\(_{10}\) of ~2.

Because Mg\(^{2+}\) must first dissociate from Mg\(^{2+}\)-EDTA (at a rate of 3/s at 10\(^\circ\)C) before Ca\(^{2+}\) can be chelated, Mg\(^{2+}\)-EDTA is an even slower Ca\(^{2+}\) chelator than EGTA. Mixing 20 \(\mu\)M Ca\(^{2+}\) with 200 \(\mu\)M EDTA and increasing [Mg\(^{2+}\)] allowed for the creation of ACTs with hws from 0.3 - 50 ms. When Mg\(^{2+}\)-EDTA is used as the Ca\(^{2+}\) chelator instead of EGTA, less Ca\(^{2+}\) must be used since the Ca\(^{2+}\) chelating potential of EDTA is compromised by the presence of increasing [Mg\(^{2+}\)].
We have used these slow Ca\(^{2+}\) chelators to create ACTs with different durations and amplitudes by mixing a specific [Ca\(^{2+}\)] with a specific concentration of EGTA or Mg\(^{2+}\)-EDTA in a stopped-flow apparatus. In this study, we have followed the response of fluorescent Ca\(^{2+}\) indicators, Ca\(^{2+}\) binding proteins and Ca\(^{2+}\) dependent enzymes which were exposed to ACTs of different duration. This has allowed us to characterize the kinetic tuning of these proteins and enzymes to Ca\(^{2+}\) transients.

Ca\(^{2+}\) indicators like MF2 and fluo-3 have very rapid (2 - 9 x 10\(^{-8}\) M\(^{-1}\)s\(^{-1}\) at 16-22\(^\circ\)C) Ca\(^{2+}\) on-rates relative to EGTA (97, 98, 99). Thus, when Ca\(^{2+}\) is mixed with these Ca\(^{2+}\) indicators in the presence of EGTA, [Ca\(^{2+}\)] will rise and bind to the indicators before EGTA. Consistent with this, both indicators underwent an increase in fluorescence during the mixing time of the instrument, followed by a decrease in their fluorescence upon Ca\(^{2+}\) chelation. Our studies show that during a 1.2 ms hw ACT, MF2 accurately reported the rate of fall in [Ca\(^{2+}\)]\(_{free}\), while fluo-3 reported the fall in [Ca\(^{2+}\)]\(_{free}\) at an ~6-fold slower rate. These results are consistent with the fact that fluo-3 has ~100-fold higher Ca\(^{2+}\) affinity and an ~70 fold slower Ca\(^{2+}\) off-rate (~370/s at 22\(^\circ\)C) compared to MF2 (~27,000/s at 22\(^\circ\)C) (83, 98, 99, 100).

Furthermore, in skeletal muscle, fluo-3 reports the rate of fall in twitch Ca\(^{2+}\) transients ~3 to 4-fold more slowly than MF2 (83, 89, 101). Thus, fluo-3 reports the rate of fall of rapid ACTs, and of real Ca\(^{2+}\) transients in living muscle, with a kinetic delay because of its slow Ca\(^{2+}\) off-rate.
The slow Ca\textsuperscript{2+} off-rate from fluo-3 gives this indicator a longer "memory" for previous Ca\textsuperscript{2+} transients by allowing the indicator to bind Ca\textsuperscript{2+} and fluoresce for a longer time after the Ca\textsuperscript{2+} transient has fallen. Unlike fluo-3, MF2 allows an accurate visualization of Ca\textsuperscript{2+} transients produced in a stopped-flow apparatus, and in living muscle, due to its faster Ca\textsuperscript{2+} on and off-rates. Fast Ca\textsuperscript{2+} indicators like MF2 have a much shorter memory of the Ca\textsuperscript{2+} transients that they experience. The slower the Ca\textsuperscript{2+} off-rate from the Ca\textsuperscript{2+} indicator (or Ca\textsuperscript{2+}-binding protein) the longer the indicator will have a memory of a rapid Ca\textsuperscript{2+} transient (and the longer the Ca\textsuperscript{2+}-binding protein will be activated after the Ca\textsuperscript{2+} transient).

Our studies with skeletal muscle TnC verified that its N-terminal regulatory sites have an ~60-fold faster Ca\textsuperscript{2+} on-rate than does EGTA. When TnC was exposed to ACTs of decreasing duration, we observed less transient occupancy of the regulatory sites. Computer simulations (see Appendix, figure 5.1) of these reactions suggested that the Ca\textsuperscript{2+} on-rate to the regulatory sites of TnC was $-8.0 \times 10^7$ M\textsuperscript{-1} s\textsuperscript{-1}, at 10°C (see table 3.1). This agrees with an earlier determination of the Ca\textsuperscript{2+} on-rates to the regulatory sites of TnC of $-1 \times 10^8$ M\textsuperscript{-1} s\textsuperscript{-1}, at 4°C (91). Thus, by subjecting a Ca\textsuperscript{2+}-binding protein to ACTs of different duration and examining the percent of the protein bound to Ca\textsuperscript{2+} as a function of ACT duration, we can accurately determine the rate of Ca\textsuperscript{2+} binding to that protein. The rapid Ca\textsuperscript{2+} on and off-rates of the regulatory sites of TnC allow this protein to bind and release Ca\textsuperscript{2+} quickly to initiate contraction and relaxation, respectively (102).
Johnson et al. (8) have previously shown when a skeletal muscle was loaded with intracellular EGTA and subjected to a twitch, Ca\(^{2+}\) could first bind to the regulatory sites of TnC to produce contraction followed by relaxation as Ca\(^{2+}\) was chelated by EGTA and resequestered by the SR. Thus, both in our in vitro stopped-flow studies described above, and also in vivo, the regulatory sites of TnC have a much more rapid Ca\(^{2+}\) on-rate than does EGTA allowing a transient occupancy of the N-terminal regulatory sites and a transient contraction.

Johnson et al. (43) have previously shown that the N-terminal of CaM exhibits \~70-fold faster rates of Ca\(^{2+}\) association than does its C-terminal (1.6\times10^8 M\(^{-1}\) s\(^{-1}\) vs 2.3\times10^6 M\(^{-1}\) s\(^{-1}\)) (see table 3.1). Furthermore, the rates of Ca\(^{2+}\) dissociation from CaM's N- and C-terminal Ca\(^{2+}\)-binding sites in the presence of RS-20 are \~200 and \~20 times slower, respectively, than the rates of Ca\(^{2+}\) dissociation from these sites in the absence of peptide (43). Our present studies indicate that Ca\(^{2+}\) dissociates from the N-terminal sites of the CaM-RS-20 complex at 2/s and that this is accompanied by peptide dissociation from the N-terminal at 2/s. Similarly, Ca\(^{2+}\) dissociates from the C-terminal sites of the CaM-RS-20 complex at 0.1/s and this is accompanied by peptide dissociation from the C-terminal at the same rate.

These results are consistent with the recent findings of Brown et al. (44) who demonstrated that high affinity peptides like WFF dissociated from the N- and C-terminal of CaM at the same rate as Ca\(^{2+}\) dissociated from these domains. Thus, our results of the dissociation of the Ca\(^{2+}\)-CaM-RS-20 complex fit into the mechanism of Path A proceeding through steps 1,5,7 as defined by Brown et al (44). In this
pathway, Ca$^{2+}$ dissociates from the faster N-terminal of CaM followed by peptide dissociation and then Ca$^{2+}$ dissociates form the slower C-terminal followed by peptide dissociation. While these kinetic studies have defined the Ca$^{2+}$ exchange rates of CaM in the presence or absence of peptide, they have not allowed investigators to observe how CaM associates and dissociates with target peptides or proteins when exposed to a Ca$^{2+}$ transient. Our present studies, with the use of ACTs, show that in response to a 1.1 ms hw ACT, only the faster N-terminal of CaM was able to significantly bind Ca$^{2+}$ and expose its hydrophobic pocket rapidly enough to bind RS-20. The C-terminal domain of CaM binds Ca$^{2+}$ too slowly to allow peptide binding following a rapid Ca$^{2+}$ transient.

When CaM-MIANS and MLCK were exposed to ACTs of various duration, we found that ~50% of CaM-MIANS could complex with MLCK during a very brief (~0.5 ms hw) ACT. Thus, CaM is able to bind to MLCK after exposure to rapid ACTs (0.2 to 1.1 ms hws). The data are consistent with the interpretation that we are observing the N-terminal of CaM-MIANS binding to MLCK since: 1) the MIANS label is covalently attached to the N-terminal (Cys 28) of CaM (74); 2) the ACTs we used are too fast to allow significant C-terminal Ca$^{2+}$ binding to occur because the C-terminal of CaM has an ~70 fold slower Ca$^{2+}$ on-rate than the N-terminal (2.3x10$^6$ M$^{-1}$ s$^{-1}$ -vs- 1.6x10$^8$ M$^{-1}$ s$^{-1}$) (43); and 3) only the N-terminal of CaM was able to bind to the smooth muscle MLCK binding peptide, RS-20, during similar rapid ACTs. The observed rate of CaM binding to MLCK at ~90/s is comparable to the rapid (65/s, at 23°C) increase in Trp fluorescence that occurs.
when CaM binds to skeletal muscle MLCK (103). Modeling of the transient binding of CaM-MIANS to MLCK (see Appendix, figure 5.2) predicted the association rate of the complex to be \(6.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}\), consistent with previously determined association rates of CaM for smooth and skeletal muscle MLCK of \(2.8 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}\) - \(4.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}\), respectively (74, 104) (see table 3.1). As EGTA dissociated the CaM-MLCK complex, we observed a dissociation rate of \(\sim 1/\text{s}\) for all the ACTs at \(10^\circ\text{C}\). This slow dissociation rate is comparable to our earlier finding that EGTA dissociates the CaM-MLCK complex at \(2 - 3/\text{s}\) at \(22^\circ\text{C}\) (74, 103).

Our data indicate that even during a rapid ACT, with a much shorter hw than those observed in smooth or skeletal muscle, CaM's N-terminal can bind \(\text{Ca}^{2+}\) quickly and then rapidly associate with MLCK. The high \(\text{Ca}^{2+}\) affinity of the C-terminal lobe of CaM complexed to CaM-binding proteins may allow the C-terminal of CaM to be bound to proteins, like MLCK, at resting concentrations of cellular \(\text{Ca}^{2+}\) (43, 44, 105). If this is the case, and since binding of both the N- and C-terminal lobes of CaM to target proteins is required for enzyme activation (34), then rapid binding of \(\text{Ca}^{2+}\) to the N-terminal sites of CaM during a \(\text{Ca}^{2+}\) transient may allow the N-terminal of CaM to bind and activate target proteins like MLCK.

Subsequently, the fall in \([\text{Ca}^{2+}]\) would cause the N-terminal of CaM to dissociate from the target protein first, since the N-terminal of CaM has an \(\sim 20\) fold faster \(\text{Ca}^{2+}\) off-rate than the C-terminal when complexed with MLCK (2/s - vs- 0.1/s (43)). Consistent with this interpretation, EGTA inactivated the CaM-MLCK complex at a rate of \(\sim 1/\text{s}\) (106). Thus, \(\text{Ca}^{2+}\) dissociation from the N-terminal sites of
CaM in the Ca\(^{2+}\)-CaM-MLCK complex is presumably responsible for enzyme inactivation as [Ca\(^{2+}\)] falls (43). This implies that the faster, lower affinity N-terminal Ca\(^{2+}\)-binding sites of CaM may be the regulatory sites of this protein, analogous to the rapid N-terminal Ca\(^{2+}\)-binding sites of TnC which regulate cardiac and skeletal muscle contraction and relaxation (107).

The SR Ca\(^{2+}\)-ATPase was half-maximally activated in a transient fashion by an ~5 ms hw ACT. The ability of the SR Ca\(^{2+}\)-ATPase to respond to rapid ACTs is consistent with a fast Ca\(^{2+}\) on-rate (~2x10\(^7\) to 4x10\(^8\) M\(^{-1}\) s\(^{-1}\)) to this enzyme (108, 109). The rate of increase in TNP-ATP fluorescence that we observe at ~83/s at 20 °C is consistent with earlier reports of Ca\(^{2+}\)-dependent enzyme activation at 85 - 150/s under conditions of saturating TNP-ATP and/or ATP at ~25°C (95, 110). It was interesting that the ACT had fallen back to baseline before the enzyme exhibited any activation. There was an ~30 ms delay between the time required for peak Ca\(^{2+}\) and peak activation of the SR Ca\(^{2+}\)-ATPase, consistent with a model of the catalytic cycle of the SR Ca\(^{2+}\)-ATPase proposed by Inesi et al. (111). This kinetic delay may provide a mechanism which would allow Ca\(^{2+}\) to bind to the N-terminal regulatory sites of TnC and initiate contraction before the SR Ca\(^{2+}\)-ATPase could be activated to resequester Ca\(^{2+}\) and facilitate relaxation.

Our rates of EGTA-induced SR Ca\(^{2+}\)-ATPase inactivation (~4/s) are comparable to previous studies in which the enzyme was inactivated by EGTA at ~1.8/s at 25°C (95). EGTA inactivates the SR Ca\(^{2+}\)-ATPase by chelating the [Ca\(^{2+}\)]\(_{free}\) and preventing the enzyme from binding more Ca\(^{2+}\). The rate of
inactivation of the SR Ca\textsuperscript{2+}-ATPase that we observe (4/s) may correspond to the rate (~3/s) at which prebound, occluded Ca\textsuperscript{2+} is translocated into the lumen of SR Ca\textsuperscript{2+}-ATPase vesicles (112). After the translocation of Ca\textsuperscript{2+}, the SR Ca\textsuperscript{2+}-ATPase inactivates since the pump can no longer bind free Ca\textsuperscript{2+} due to EGTA chelation. These studies are consistent with our observed decrease in TNP-ATP fluorescence occurring at the rate of SR luminal Ca\textsuperscript{2+} release. Our data are consistent with theories which suggest that as soon as Ca\textsuperscript{2+} is released in a skeletal muscle cell, the SR Ca\textsuperscript{2+}-ATPase can initially bind Ca\textsuperscript{2+}, and with a kinetic delay, actively begin to sequester Ca\textsuperscript{2+} (109).

We have exposed several Ca\textsuperscript{2+} indicators, Ca\textsuperscript{2+}-binding proteins and a Ca\textsuperscript{2+}-dependent enzyme to ACTs of various durations and observed their transient binding of Ca\textsuperscript{2+}, complex formation and activation. This has allowed us to characterize the response of these Ca\textsuperscript{2+}-dependent proteins to Ca\textsuperscript{2+} transients of varying amplitude and duration. We show that their response to and their "memory" of these Ca\textsuperscript{2+} transients are dictated by their Ca\textsuperscript{2+} exchange kinetics. Proteins with higher Ca\textsuperscript{2+} affinity and slower Ca\textsuperscript{2+} off-rates exhibit a longer memory for Ca\textsuperscript{2+} and can remain active long after the Ca\textsuperscript{2+} transients have subsided. Furthermore, by characterizing the response of these proteins to ACTs, we can predict with greater certainty, how they would respond to natural Ca\textsuperscript{2+} transients to regulate cellular phenomena.

Future applications of these ACTs should further increase our understanding of how different Ca\textsuperscript{2+}-dependent phenomena are controlled by cellular Ca\textsuperscript{2+} transients and by the Ca\textsuperscript{2+} tuning of Ca\textsuperscript{2+}-binding proteins and enzymes.
3.4 Conclusions

1. Using EGTA and EDTA+Mg\(^{2+}\) it is possible to create artificial Ca\(^{2+}\) transients with half widths from 0.1 to 50ms.

2. During a 1.2 ms half width artificial Ca\(^{2+}\) transient, mag-fura-2 accurately reports both the rapid rise and fall in Ca\(^{2+}\), while fluo-3 reports an ~6-fold slower rate of fall in Ca\(^{2+}\).

3. Exposing the regulatory N-terminal Ca\(^{2+}\)-binding sites of troponin C to artificial Ca\(^{2+}\) transients of varying duration confirms a Ca\(^{2+}\) on-rate of \(-8.0 \times 10^7\) M\(^{-1}\)s\(^{-1}\).

4. During a 1.1 ms half width artificial Ca\(^{2+}\) transient, only the N-terminal of CaM can rapidly bind Ca\(^{2+}\), expose its hydrophobic pocket and bind RS-20, confirming the N-terminals Ca\(^{2+}\) on-rate of \(-1.6 \times 10^8\) M\(^{-1}\)s\(^{-1}\).

5. During artificial Ca\(^{2+}\) transients, as brief as 0.2 ms, calmodulin’s N-terminal can transiently bind to myosin light chain kinase.

6. An artificial Ca\(^{2+}\) transient, as brief as 1.8 ms, produced significant transient activation of the SR-Ca\(^{2+}\)-ATPase.

7. Artificial Ca\(^{2+}\) transients are useful tools for determining how Ca\(^{2+}\) indicators, Ca\(^{2+}\)-binding proteins and Ca\(^{2+}\)-dependent enzymes respond to Ca\(^{2+}\) transients of varying duration.
CHAPTER 4

ROLE OF ACID PAIRS IN CALCIUM AND MAGNESIUM BINDING AND EXCHANGE WITH THE N-TERMINAL CALCIUM BINDING SITES OF CALMODULIN

4.1 Introduction

CaM is a ubiquitous eukaryotic Ca\(^{2+}\)-binding protein that contains two EF-hand Ca\(^{2+}\)-binding sites in both its N- and C-terminal globular domains (for review 16). CaM has a dumbbell shape with its N- and C-terminal domains separated by a 28 residue flexible central helix. Upon Ca\(^{2+}\) binding, each domain exposes a hydrophobic pocket which allows CaM to bind and activate greater than 30 different target enzymes (for review 16). The N-terminal Ca\(^{2+}\)-binding sites of CaM have \(~3\)-6-fold lower Ca\(^{2+}\) affinity (\(~3\) - 10 mM) than the C-terminal Ca\(^{2+}\)-binding sites (\(~1\) - 3 mM) (42, 43). The lower Ca\(^{2+}\) affinity of the N-terminal of CaM is reflected in the fact that it has an approximately 80-170-fold faster Ca\(^{2+}\) dissociation rate (43 - 45), but with an approximately 70-fold faster Ca\(^{2+}\) association rate (43). Since the N-terminal of CaM has faster Ca\(^{2+}\) binding and dissociation kinetics, it may be the regulatory domain of CaM similar in function to the N-terminal of TnC (43). CaM was originally thought to contain four Ca\(^{2+}\) specific sites, but recent NMR studies
have shown that Ca\textsuperscript{2+}-binding site I of CaM can bind Mg\textsuperscript{2+} with an affinity (~1mM) (113, 114) in the range of the free [Mg\textsuperscript{2+}] \textit{in vivo} (0.5 - 2 mM) (115). Interestingly, Mg\textsuperscript{2+} binding does not cause the opening of the hydrophobic pockets of CaM or allow activation of enzymes which is the hallmark of Ca\textsuperscript{2+} binding to CaM (113, 114, 116 - 118). Because the Ca\textsuperscript{2+} binding properties of CaM have been well characterized, this universal Ca\textsuperscript{2+}-binding protein provides an excellent model for determining the fundamental structural elements that control cation binding and specificity in EF-hand proteins (58, 62, 63).

CaM and more than 400 other Ca\textsuperscript{2+}-binding proteins use the EF-hand motif to bind Ca\textsuperscript{2+} and/or Mg\textsuperscript{2+}. The EF-hand consists of a 29 amino acid helix-loop-helix secondary structure, first described by Kretsinger and Nockolds (46) in the crystal structure of carp parvalbumin. The first 9 amino acids form an α-helix, followed by a 12 residue Ca\textsuperscript{2+}-binding loop whose last three residues blend into a final 8 residue α-helix. The Ca\textsuperscript{2+}-binding loop domain of the EF-hand coordinates Ca\textsuperscript{2+} binding through six chelating residues which donate 7 oxygen atoms. These oxygen atoms are geometrically arranged on the axes of a pentagonal bipyramid at positions 1 (+x), 3 (+y), 5 (+z), 7 (-y), 9 (-x) and 12 (-z) (see Fig 1.8). Glu almost invariably occupies the -z position and donates two oxygens through its bidentate carboxylate sidechain. Ca\textsuperscript{2+}-binding proteins typically contain at least two coupled EF-hands which often bind Ca\textsuperscript{2+} and/or Mg\textsuperscript{2+} in a cooperative manner (for review 9, 16, 48, 49).
Less than 5% of the known EF-hand Ca\(^{2+}\)-binding sites have been evaluated for their Ca\(^{2+}\) affinity and specificity (9). These studies suggest that EF-hand Ca\(^{2+}\) affinity can vary by 10\(^6\)-fold and Ca\(^{2+}\) dissociation and association rates can vary by 10\(^3\)-fold (9, 48, 49). Multiple factors tune the Ca\(^{2+}/Mg^{2+}\) affinities, selectivity and kinetics in different EF-hand proteins. Ultimately, the free energy differences between the apo protein plus solvated cation and the cation-protein complex dictate ion binding properties (49, 55, 119). This global theme can be influenced locally by structural elements of the EF-hand, including chelating and non-chelating residues within the Ca\(^{2+}\)-binding loop and the hydrophobicity of the two flanking helices (55, 56, 63, 120 - 123). Arrangement and number of negatively charged residues within or near the Ca\(^{2+}\)-binding loop can also modify the affinity and kinetics of Ca\(^{2+}/Mg^{2+}\) binding (57, 58, 62 - 64, 66, 124, 125).

Reid and Hodges (60) proposed the acid pair hypothesis which relates the location and number of negatively charged residues at chelating positions with Ca\(^{2+}\) affinity. This hypothesis predicts that the highest Ca\(^{2+}\) affinity should occur in a single EF-hand when there are a maximum of four acidic residues in chelating positions 1, 3, 5, 9 or 12 causing pairing of the carboxylate oxygen atoms on the pentagonal bipyramid axes at the +x,-x (X acid pair) and +z,-z (Z acid pair) positions. Introduction of a fifth carboxylate into the Ca\(^{2+}\)-binding loop was predicted to decrease Ca\(^{2+}\) affinity due to increased electrostatic repulsion (64). The third EF-hand of CaM has three acidic residues but no acid pairs. Reid and coworkers (61) have shown that Ca\(^{2+}\) affinity was increased ~12-fold when the
number of acid pairs were increased from zero to one and ~39-fold when the acid pairs were increased from zero to two in synthetic EF-hand peptides based on CaM's third Ca\(^{2+}\)-binding site. Only synthetic EF-hand peptides which contained a Z acid pair exhibited Mg\(^{2+}\) binding (72, 73). Recently, Wu and Reid (62) have used cassette-mediated mutagenesis in CaM to demonstrate an ~160-fold increase in Ca\(^{2+}\) affinity in the third EF-hand of CaM when the number of its acid pairs were increased from zero to two and aligned on the X and Z axes. In these studies, the fourth Ca\(^{2+}\)-binding site of CaM was inactivated by a D133E mutation at the +z position producing an ~24-fold decrease in Ca\(^{2+}\) affinity at the third Ca\(^{2+}\)-binding site (126). These studies show that dramatic increases in Ca\(^{2+}\) affinity can occur in synthetic EF-hand peptides and EF-hand proteins by solely increasing the number of acid pairs.

Wild type CaM (wtCaM) has a Z acid pair in Ca\(^{2+}\)-binding site I and a X acid pair in Ca\(^{2+}\)-binding site II. We explored the effects that number and location of acid pairs had on Ca\(^{2+}\) and Mg\(^{2+}\) affinity and exchange rates in the N-terminal of CaM. Using site-directed mutagenesis we changed the number of acid pairs in Ca\(^{2+}\)-binding site I from zero to three when site II was unmodified or mutated to prevent Ca\(^{2+}\) binding to site II. We also changed the number of acid pairs in site II from zero to three while leaving site I unmodified. Phe 19 was mutated to Trp in some of the CaM mutants to produce an intrinsic fluorescent probe for following Ca\(^{2+}\)/Mg\(^{2+}\) binding to the N-terminal of CaM. This produced a series of mutant CaM's with ~560-fold difference in N-terminal Ca\(^{2+}\) affinities and allowed us to determine the
effect of number and location of acid pairs on Ca\(^{2+}\) affinity, selectivity and exchange in the N-terminal of CaM. Mutant CaMs that exhibited the widest range in N-terminal Ca\(^{2+}\) affinity were examined for their Ca\(^{2+}\)-dependent activation of phosphodiesterase.

4.2 Results

4.2.1 Ca\(^{2+}\) binding to site I acid pair mutants when Ca\(^{2+}\)-binding site II was impaired

Wu and Reid have shown that increasing the number of acid pairs in the third EF-hand of CaM produces a dramatic increase in C-terminal Ca\(^{2+}\) affinity when the fourth EF-hand was inactivated (62). In an effort to see if their C-terminal results would also apply to the N-terminal of CaM, we changed the number of acid pairs in Ca\(^{2+}\)-binding site I from zero to three, when Ca\(^{2+}\) binding to site II was impaired by an E67A mutation at the -z residue. These mutants also contained a F19W mutation which introduced an intrinsic fluorescent probe, allowing us to follow N-terminal Ca\(^{2+}\) binding by an increase in Trp fluorescence. The following nomenclature is used to describe these mutant CaMs: W- representing a CaM mutant with a F19W mutation; I and II, representing the first and second N-terminal EF-hands of CaM; X, Y and Z representing the acid pairs that are present in each Ca\(^{2+}\)-binding loop with O meaning no acid pairs; and (no-z) representing a Ca\(^{2+}\)-binding loop in which the -z Glu chelating residue has been mutated to Ala. For instance, wtCaM is represented by I\(_2\)II\(_X\) since the first and second EF-hands contain a Z and X acid pair, respectively.
Figure 4.1A shows the Ca\(^{2+}\) induced increase in N-terminal Trp fluorescence upon Ca\(^{2+}\) binding to W-I\(_2\)II\(_X\), W-I\(_X\), W-I\(_Y\), W-I\(_Z\), W-I(y,z), W-Io II\(_X\) and W-Io II\(_X\). These proteins were half maximally saturated at pCa 5.4 for W-I\(_2\)II\(_X\), 4.5 for W-I\(_X\), 3.3 for W-I\(_Y\), 3.2 for W-I\(_Z\), 3.1 for W-Io II\(_X\) and 2.9 for and W-Io II\(_X\). These data show that when Ca\(^{2+}\) binding to site II was impaired by the E67A mutation (W-I\(_Z\)II\(_X\)) there was an ~122-fold decrease in N-terminal Ca\(^{2+}\) affinity compared to the parent W-I\(_2\)II\(_X\) construct.

Removal of the endogenous Z acid pair in site I (W-Io II\(_X\)) decreased Ca\(^{2+}\) affinity another 2.3-fold. Figure 4.1B compares the ratio of Ka values of all these mutants with respect to the Ka of the mutant with no acid pairs in site I, W-Io II\(_X\) (see table 4.1, Tryptophan N-Term Kd). These data indicate that as the number of acid pairs in site I were increased from zero to one at any position (X, Y or Z) or to two with any combination (X,Y; X,Z or Y,Z) that there was only an ~2-fold increase in N-terminal Ca\(^{2+}\) affinity. However, when three acid pairs were introduced into site I (W-I\(_X\), Y, Z\(_X\)) there was an ~40-fold increase in Ca\(^{2+}\) affinity. Thus, increasing the number of acid pairs in site I from zero to three produced a dramatic increase in N-terminal Ca\(^{2+}\) affinity when Ca\(^{2+}\) binding to site II was impaired.
Figure 4.1: Ca\(^{2+}\) binding to site I acid pair mutants with an inactivated site II.

Figure 4.1A shows the Ca\(^{2+}\)-dependent increase in Trp fluorescence for W-I\(_2\)II\(_X\) (■), W-I\(_X\)Y\(_2\)II\(_X(\alpha-\alpha)\) (●), W-I\(_2\)II\(_X(\alpha-\alpha)\) (□), W-I\(_Y\)Z\(_2\)II\(_X(\alpha-\alpha)\) (○), W-I\(_O\)II\(_X(\alpha-\alpha)\) (□) and W-I\(_O(\alpha-\alpha)\)II\(_X\) (▲) shown as a function of pCa. Increasing concentrations of Ca\(^{2+}\) were added to 1 mL of each CaM (500 nM) in 200 mM MOPS, 90 mM KCl, 2 mM EGTA, pH 7.0 at 22°C to yield the indicated pCa. Tryptophan fluorescence was monitored at 335 nm with excitation at 275 nm. 100% fluorescence corresponds to a 2.8, 1.2, 1.3, 1.2 and 1.3-fold increase in tryptophan fluorescence for W-I\(_2\)II\(_X\), W-I\(_X\)Y\(_2\)II\(_X(\alpha-\alpha)\), W-I\(_2\)II\(_X(\alpha-\alpha)\), W-I\(_Y\)Z\(_2\)II\(_X(\alpha-\alpha)\), W-I\(_O\)II\(_X(\alpha-\alpha)\) and W-I\(_O(\alpha-\alpha)\)II\(_X\), respectively. Each data point represents an average of at least 3 titrations ± S.E. fit with a sigmoidal curve. Figure 4.1B compares the ratio of Ka values for all the site I acid pair mutants, when site II was inactivated, with respect to the Ka of the mutant with no acid pairs in site I, W-I\(_O\)II\(_X(\alpha-\alpha)\). Each bar represents a mutant with 0, 1, 2 or 3 acid pairs in site I and labeled accordingly to the position of the added acid pair(s). For example, W-I\(_2\)II\(_X(\alpha-\alpha)\) is in group 1, column Z since it has only a single Z acid pair in site I. W-I\(_O\)II\(_X(\alpha-\alpha)\) was half-maximally saturated with Ca\(^{2+}\) at 1.24 mM.
Figure 4.1: Ca^{2+} binding to site I acid pair mutants with an inactivated site II.
<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Mutant Protein</th>
<th>Site I Acid Pairs</th>
<th>Site II Acid Pairs</th>
<th>Tryptophan N-Term Kd, Hill Coef</th>
</tr>
</thead>
<tbody>
<tr>
<td>W-I_{D(No-Z)}II_{X}</td>
<td>F19W E31A</td>
<td>none</td>
<td>X</td>
<td>895 uM, 0.9</td>
</tr>
<tr>
<td>W-I_{D}II_{X(No-Z)}</td>
<td>F19W D24N E67A</td>
<td>none</td>
<td>X</td>
<td>1.24 mM, 1.2</td>
</tr>
<tr>
<td>W-I_{X}II_{X(No-Z)}</td>
<td>F19W D24N T26D E67A</td>
<td>X</td>
<td>X</td>
<td>606 uM, 1.3</td>
</tr>
<tr>
<td>W-I_{Y}II_{X(No-Z)}</td>
<td>F19W D24N T26D E67A</td>
<td>Y</td>
<td>X</td>
<td>794 uM, 0.8</td>
</tr>
<tr>
<td>W-I_{Z}II_{X(No-Z)}</td>
<td>F19W E67A</td>
<td>Z</td>
<td>X</td>
<td>545 uM, 8.8</td>
</tr>
<tr>
<td>W-I_{XY}II_{X(No-Z)}</td>
<td>F19W D24N T26D T28D E67A</td>
<td>X,Y</td>
<td>X</td>
<td>781 uM, 1.3</td>
</tr>
<tr>
<td>W-I_{XZ}II_{X(No-Z)}</td>
<td>F19W T28D E67A</td>
<td>X,Z</td>
<td>X</td>
<td>576 uM, 1.3</td>
</tr>
<tr>
<td>W-I_{YZ}II_{X(No-Z)}</td>
<td>F19W T26D E67A</td>
<td>Y,Z</td>
<td>X</td>
<td>584 uM, 1.1</td>
</tr>
<tr>
<td>W-I_{XYZ}II_{X(No-Z)}</td>
<td>F19W T26D T28D E67A</td>
<td>X,Y,Z</td>
<td>X</td>
<td>33 uM, 0.7</td>
</tr>
</tbody>
</table>

Table 4.1: Comparison of the Ca²⁺ affinities for site 1 acid pair mutants with an inactivated site II.
4.2.2 \( \text{Ca}^{2+} \) binding to site I acid pair mutants with a functional \( \text{Ca}^{2+} \)-binding site II

The above studies showed that increasing the number of acid pairs in site I increased \( \text{Ca}^{2+} \) affinity when site II was impaired by the E67A mutation. Next we determined the effect of increasing site I acid pairs from zero to three when site II was unmodified (one X acid pair). Figure 4.2A shows \( \text{Ca}^{2+} \) binding to \( I_{X,Y,Z}^{X} \), \( I_{Y,Z}^{X} \), \( I_{Z}^{X} \) and \( I_{0}^{X} \) determined by \( \text{Ca}^{2+} \) competition binding assays using the chromophoric \( \text{Ca}^{2+} \) chelator BAPTA (4). All of these CaMs bound four moles of \( \text{Ca}^{2+} \) per mole protein. Two moles of \( \text{Ca}^{2+} \) bound to the N-terminal domains of \( I_{X,Y,Z}^{X} \), \( I_{Y,Z}^{X} \), \( I_{Z}^{X} \) and \( I_{0}^{X} \) with global \( \text{Ca}^{2+} \) dissociation constants (Kd) of 7.4, 6.4, 9.3 and 33.8 \( \mu \text{M} \), respectively (see table 2, Bapta N-Term Global Kd).

Removal of the endogenous Z acid pair from \( \text{Ca}^{2+} \)-binding site I (I\( _{0}^{X} \)) produced an \(-3.6\)-fold decrease in N-terminal global \( \text{Ca}^{2+} \) affinity compared to wtCaM (I\( _{2}^{X} \)). Figure 4.2B compares the ratio of \( \text{K}_a \) values of all these mutants with respect to the \( \text{K}_a \) of the mutant with no site I acid pairs, I\( _{0}^{X} \). These data indicate that nearly all of the loss in N-terminal \( \text{Ca}^{2+} \) affinity of I\( _{0}^{X} \) could be restored when the number of acid pairs in site I were increased from zero to one at any position (X, Y or Z), when site II was unmodified. Increasing the number of acid pairs in site I to two and then three had little further effect on global N-terminal \( \text{Ca}^{2+} \) affinity.

F19W was introduced into the site I acid pair mutants described above in order to directly follow \( \text{Ca}^{2+} \) binding to the N-terminal \( \text{Ca}^{2+} \)-binding sites of these
mutants. Figure 4.3 shows the Ca\(^{2+}\) induced increase in N-terminal Trp fluorescence upon Ca\(^{2+}\) binding to W- I\(_{X,Y,Z}II_{X}\), W- I\(_{Y,Z}II_{X}\), W- I\(_{Z}II_{X}\) and W- I\(_{O}II_{X}\). These proteins were half-maximally saturated at pCa 5.6 for W- I\(_{X,Y,Z}II_{X}\), 5.6 for W- I\(_{Y,Z}II_{X}\), 5.4 for W- I\(_{Z}II_{X}\) and 4.6 for W- I\(_{O}II_{X}\). Table 4.2 (Tryptophan N-Term Kd) shows the results for these mutants and the other Trp site I mutants not shown in figure 4.3. These results show that removal of the endogenous Z acid pair in site I decreased Ca\(^{2+}\) affinity ~5-fold compared to W- I\(_{Z}II_{X}\). Furthermore, increasing the number of acid pairs from zero to one at any position (X, Y or Z) produced nearly full recovery of the loss in Ca\(^{2+}\) affinity that was observed with removal of the endogenous Z acid pair (W- I\(_{O}II_{X}\). Addition of two or three acid pairs produced little additional increase in Ca\(^{2+}\) affinity. These results with the F19W site I mutants were similar to the BAPTA results in terms of relative change in Ca\(^{2+}\) affinity, in spite of an ~2-fold increase in Ca\(^{2+}\) affinity caused by the introduction of F19W. They confirm that any single acid pair in site I was capable of supporting high Ca\(^{2+}\) affinity to the N-terminal of CaM.
Fig 4.2: Ca$^{2+}$ binding to site I acid pair mutants with a functional Ca$^{2+}$-binding site II.

Figure 4.2A shows the plot of the calculated number of moles Ca$^{2+}$ bound to I$_{X,Y,Z}$ (▲), I$_{Y,Z}$ (O), I$_{Z}$ (■) and I$_{O}$ (●) per mole protein as a function of added Ca$^{2+}$ using a chromophoric Ca$^{2+}$ chelator competition binding assay. Ca$^{2+}$ binding to each protein was determined by adding increasing concentrations of Ca$^{2+}$ to 1 mL solution of 30 μM protein and 30 μM 5-5'-Br$_2$BAPTA in 10 mM MOPS, 90 mM KCl, pH 7.0 at 22°C and following the decrease in 5-5'-Br$_2$BAPTA absorption at 263 nm. Calibration curves were performed by conducting the same Ca$^{2+}$ titrations in an identical solution without protein. These titrations were conducted and analyzed as previously described by Linse et al. (42). The data analysis program, which provides least squares fits to the titrations to obtain macroscopic binding constants was generously provided by Sara Linse of Lund University, Lund Sweden. Protein solutions and buffers were rendered Ca$^{2+}$ free by incubation with Chelex-100. All titrations were done on a Beckman DU spectrophotometer. Figure 4.2B compares the ratio of Ka values for all the site I acid pair mutants, when site II was native, with respect to the Ka of the mutant with no site I acid pairs, I$_{O}$II$X$. I$_{O}$II$X$ was half-maximally saturated with Ca$^{2+}$ at 33.8 μM.
Figure 4.2: Ca^{2+} binding to site I acid pair mutants with a functional Ca^{2+}-binding site II.
Figure 4.3: Ca\(^{2+}\) binding to W-site I acid pair mutants with a functional Ca\(^{2+}\)-binding site II.

The figure shows the Ca\(^{2+}\) dependent increase in Trp fluorescence for W-\textit{I}_X\textit{Y}_Z\Pi_X (\textcolor{red}{\triangle}), \textit{W-I}_Y\textit{Z}\Pi_X (\textcolor{blue}{O}), \textit{W-I}_Z\Pi_X (\textcolor{green}{■}) and \textit{W-I}_O\Pi_X (\textcolor{black}{●}) as a function of pCa. Increasing concentrations of Ca\(^{2+}\) were added to 1 mL of each CaM (1 μM) in 200 mM MOPS, 90 mM KCl, 2 mM EGTA, pH 7.0 at 22°C to yield the indicated pCa. Tryptophan fluorescence was monitored at 335 nm with excitation at 275 nm. 100% fluorescence corresponds to a 2.8, 2.9, 2.8 and 2.7-fold increase in tryptophan fluorescence for \textit{W-I}_X\textit{Y}_Z\Pi_X, \textit{W-I}_Y\textit{Z}\Pi_X, \textit{W-I}_Z\Pi_X and \textit{W-I}_O\Pi_X, respectively. Each data point represents an average of 3 titrations ± S.E. fit with a sigmoidal curve.
Table 4.2: Comparison of the Ca^{2+} affinities, dissociation rates and association rates of the site I and site II acid pair mutants. NA represents data that could not be calculated.
<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Mutant Protein</th>
<th>Site I Acid Pair</th>
<th>Site II Acid Pair</th>
<th>Tryptophan N-Term Kₜ, Hill Coef</th>
<th>Tryptophan N-Term Off Rate (s⁻¹)</th>
<th>Tryptophan N-Term On Rate (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>W₁₋₁₀ₓₓₓₓ</td>
<td>F₁₉ W</td>
<td>Z X</td>
<td></td>
<td>4.45 ± 0.08 μM, 1.9 ± 1</td>
<td>248 ± 15</td>
<td>5.6 ± 0.7</td>
</tr>
<tr>
<td>W₁₋₁₀ₓₓₓₓ</td>
<td>F₁₉ W D₂₄ N</td>
<td>none</td>
<td>X</td>
<td>22.6 ± 0.9 μM, 1.3 ± 1</td>
<td>155 ± 2</td>
<td>6.9 ± 0.6</td>
</tr>
<tr>
<td>W₁₋₁₀ₓₓₓₓ</td>
<td>F₁₉ W D₂₄ N T₂₈ D</td>
<td>X X</td>
<td></td>
<td>7.0 ± 0.4 μM, 1.2 ± 1</td>
<td>23 ± 0.2</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>W₁₋₁₀ₓₓₓₓ</td>
<td>F₁₉ W D₂₄ N T₂₆ D</td>
<td>Y X</td>
<td></td>
<td>6.1 ± 0.3 μM, 1.6 ± 1</td>
<td>150 ± 2</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>W₁₋₁₀ₓₓₓₓ</td>
<td>F₁₉ W D₂₄ N T₂₆ D</td>
<td>X,Y</td>
<td></td>
<td>2.8 ± 0.2 μM, 1.4 ± 1</td>
<td>42 ± 0.3</td>
<td>1.5 ± 0.7</td>
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<tr>
<td>W₁₋₁₀ₓₓₓₓ</td>
<td>F₁₉ W T₂₈ D</td>
<td>X,Z</td>
<td></td>
<td>2.73 ± 0.02 μM, 2.2 ± 1</td>
<td>297 ± 6</td>
<td>1.1 ± 0.6</td>
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<tr>
<td>W₁₋₁₀ₓₓₓₓ</td>
<td>F₁₉ W T₂₆ D</td>
<td>Y,Z</td>
<td></td>
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<td>4.9 ± 0.7</td>
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<tr>
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<td>X,Y,Z</td>
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<td>3.0 ± 0.5</td>
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<td></td>
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<td>F₁₉ W N₆₀ D D₆₄ N</td>
<td>Z Z</td>
<td></td>
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<td>7.3 ± 0.7</td>
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<td>Z,X,Z</td>
<td></td>
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<td>391 ± 3</td>
<td>1.5 ± 0.6</td>
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<tr>
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<td>Z Y,Z</td>
<td></td>
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<td>2.6 ± 0.6</td>
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<tr>
<td>W₁₋₁₀ₓₓₓₓ</td>
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<td>Z,X,Y,Z</td>
<td></td>
<td>4.1 ± 0.1 μM, 2.1 ± 0.3</td>
<td>1110 ± 26</td>
<td>2.7 ± 0.6</td>
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</tbody>
</table>
4.2.3 Ca$^{2+}$ binding to site II acid pair mutants with a functional Ca$^{2+}$-binding site I

The above studies showed that a single acid pair in site I was sufficient to support high N-terminal Ca$^{2+}$ affinity when site II was unmodified. Next we determined the effect of changing site II acid pairs from zero to three when site I was unmodified (one Z acid pair). The results with these mutants are shown in table 4.2 (Bapta N-Term Global Kd) and in the histogram in figure 4.4. Removal of the endogenous X acid pair in site II (I$_2$II$_0$) reduced N-terminal Ca$^{2+}$ affinity $>$25-fold compared to wtCaM (I$_2$II$_x$). Introduction of a single Y acid pair increased N-terminal Ca$^{2+}$ affinity only $\sim$10-fold compared to the 27 and 32-fold increases observed with the addition of a single X or Z acid pair, respectively. Increasing the number of acid pairs from one to two and then to three produced little additional increase in Ca$^{2+}$ affinity. Similar results were observed when the number of acid pairs was increased from zero to three in the W-site II mutants, while leaving site I unmodified (see table 4.2, Tryptophan N-Term Kd). Thus, removal of the single X acid pair in site II produces a much larger decrease in N-terminal Ca$^{2+}$ affinity than removal of the single Z acid pair in site I. Furthermore, while introduction of a single acid pair at any position in site I restored Ca$^{2+}$ affinity, much larger increases in Ca$^{2+}$ affinity were restored with the introduction of an X or Z acid pair in site II.
Figure 4.4: Comparison of Ka values for the site II acid pair mutants with a functional site I.

The histogram compares the ratio of Ka values for all the site II acid pair mutants, when site I was native, with respect to the Ka of the mutant with no site II acid pairs, I\(_2\)I\(_O\). The data for the figure were obtained as described in the figure legend of figure 4.2. I\(_2\)I\(_O\) was half-maximally saturated with Ca\(^{2+}\) at ~250 \(\mu\)M.
4.2.4 Ca$^{2+}$ exchange rates of site I acid pair mutants with a functional Ca$^{2+}$ binding site II

We were interested in determining if the increase in Ca$^{2+}$ affinity that occurred with the site I mutants resulted from an increase in Ca$^{2+}$ association rate or a decrease in Ca$^{2+}$ dissociation rate. Fluorescence stopped-flow measurements were conducted using Quin-2 to determine the rates of Ca$^{2+}$ dissociation from the site I mutants with an unmodified site II. Figure 4.5A shows the rates of Ca$^{2+}$ dissociation from the N-terminal Ca$^{2+}$-binding sites of I$_{X,Y,Z;IIx}$, I$_{Y,Z;IIx}$, I$_{Z;IIx}$ and I$_{O;IIx}$ as monitored by an increase in Quin-2 fluorescence. Approximately two moles of Ca$^{2+}$ dissociated from the N-terminal sites of I$_{X,Y,Z;IIx}$, I$_{Y,Z;IIx}$, I$_{Z;IIx}$ and I$_{O;IIx}$ at rates of 465, 283, 558 and 641 s$^{-1}$, respectively. Figure 4.5B compares the ratio of the Ca$^{2+}$ dissociation rates from the site I mutants with respect to the Ca$^{2+}$ dissociation rate of I$_{O;IIx}$ (see table 4.2, Quin-2 N-term Off Rate). Only I$_{X;IIx}$, I$_{X,Y;I}$ and I$_{Y,Z;IIx}$ had a marked effect on decreasing the N-terminal Ca$^{2+}$ dissociation rate. These mutants decreased Ca$^{2+}$ dissociation ~4.5-fold for I$_{X;IIx}$, 2.1-fold for I$_{X,Y;I}$ and 2.3-fold for I$_{Y,Z;IIx}$ compared to I$_{O;IIx}$. Of these three mutants, only the ~4.5-fold decrease in the Ca$^{2+}$ dissociation rate of I$_{X;IIx}$ could completely explain its ~4-fold increase in Ca$^{2+}$ affinity. Since I$_{O;IIx}$ had a similar Ca$^{2+}$ dissociation rate as wtCaM (I$_{Z;IIx}$) and the remaining site I mutants, the increase in N-terminal Ca$^{2+}$ affinity observed with the increased number of acid pairs in the site I mutants must be due to an increase in their Ca$^{2+}$ association rates.
Figure 4.6 shows the rates of the EGTA induced decrease in Trp fluorescence that occurs upon Ca\textsuperscript{2+} removal from the W-Site I mutants. Ca\textsuperscript{2+} dissociated from the N-terminal of these proteins at rates of 153 s\textsuperscript{-1} for W- I\textsubscript{X,Y,Z}II\textsubscript{X}, 116 s\textsuperscript{-1} for W- I\textsubscript{Y,Z}II\textsubscript{X}, 248 s\textsuperscript{-1} for W- I\textsubscript{Z}II\textsubscript{X} and 155 s\textsuperscript{-1} for W- I\textsubscript{O}II\textsubscript{X}. The relative N-terminal Ca\textsuperscript{2+} dissociation rates of the W-site I mutants (see table 4.2, Tryptophan N-Term Off Rate) were consistent with the Ca\textsuperscript{2+} dissociation rate data using Quin-2 fluorescence in figures 4.5A and 4.5B. The rates reported by the decrease in Trp fluorescence followed the true rate of Ca\textsuperscript{2+} dissociation from these mutants as was observed when Ca\textsuperscript{2+} was removed from these proteins with Quin-2 (data not shown). The Trp data support the conclusions from the Quin-2 histogram (figure 4.5B) and suggest that the increase in Ca\textsuperscript{2+} affinity that occurs with increasing the number of acid pairs in site I cannot generally be explained by a slower rate of Ca\textsuperscript{2+} dissociation. Thus, the increase in Ca\textsuperscript{2+} affinity must be due to an increase in the Ca\textsuperscript{2+} association rates.

Knowing the N-terminal Ca\textsuperscript{2+} affinity (K\textsubscript{d}) of the wtCaM site I mutants from the BAPTA studies and their Ca\textsuperscript{2+} dissociation rates (k\textsubscript{off}) from the Quin-2 experiments, we calculated the Ca\textsuperscript{2+} association rates (k\textsubscript{on}) of these mutants using the relationship k\textsubscript{on} = k\textsubscript{off} / K\textsubscript{d}. Figure 4.7 compares the ratio of the Ca\textsuperscript{2+} association rates of all the site I mutants with respect to the Ca\textsuperscript{2+} association rate of I\textsubscript{O}II\textsubscript{X} (see table 2, Quin-2/Bapta N-Term On Rate). The Ca\textsuperscript{2+} association rate increased for all of the site I mutants compared to I\textsubscript{O}II\textsubscript{X}, except for I\textsubscript{X}II\textsubscript{X}. The data suggest that an increase in the N-terminal Ca\textsuperscript{2+} association rate was the primary cause of the increase in Ca\textsuperscript{2+} affinity of the site I mutants. For example, I\textsubscript{X,Z}II\textsubscript{X} had
an ~3.6-fold faster Ca$^{2+}$ association rate than $I_0Pi_X$ and exhibited an ~3.3-fold higher Ca$^{2+}$ affinity. The same observations were seen for the W-site I mutants (see table 4.2, Tryptophan N-Term On Rate). Thus, the primary cause of the increase in Ca$^{2+}$ affinity when the number of acid pairs was increased in site I was due to an increase in the Ca$^{2+}$ association rate.
Figure 4.5: Ca$^{2+}$ dissociation from site I acid pair mutants with a functional site II.

Figure 4.5A shows the increase in Quin-2 fluorescence associated with Ca$^{2+}$ dissociation from the N-terminal sites of I$_{X,Y,Z}$II$_{X}$, I$_{Y,Z}$II$_{X}$, I$_{Z}$II$_{X}$ and I$_{0}$II$_{X}$. Each CaM (8 μM) + Ca$^{2+}$ (60 μM) in 20 mM Hepes, pH 7.0 was rapidly mixed with an equal volume of Quin-2 (150 μM) in the same buffer at 10°C. Quin-2 fluorescence was monitored through a 510 nm broad bandpass filter (Oriel, Stanford, CT) with excitation at 330 nm. Figure 4.5B compares the ratio of the Ca$^{2+}$ dissociation rates of all the site I mutants, with a native site II, with respect to the Ca$^{2+}$ dissociation rate of I$_{0}$II$_{X}$. Ca$^{2+}$ dissociated from I$_{0}$II$_{X}$ at a rate of ~641/s.
Figure 4.6: Ca$^{2+}$ dissociation from the W-site I acid pair mutants with a functional
site II.

The figure shows the decrease in tryptophan fluorescence associated with
Ca$^{2+}$ dissolution from the N-terminal sites of W-I$_{y}$, W-I$_{y}$z, W-I$_{z}$, and W-
I$_{o}$z. Each CaM (4 μM) + Ca$^{2+}$ (200 μM) in 20 mM Hepes, pH 7.0 was rapidly
mixed with an equal volume of EGTA (10 mM) in the same buffer at 10°C. CaM
tryptophan fluorescence was monitored through a UV transmitting black glass filter
(UG1 from Oriel (Stanford, CT)) with excitation at 275 nm. Each trace in 4A and
4B is an average of 5 traces fit with a single exponential equation (variance <
2.0x10$^{-4}$).
Figure 4.7: Comparison of the Ca$^{2+}$ association rates of the site I acid pair mutants with a functional site II.

The histogram compares the ratio of the Ca$^{2+}$ association rates for all the site I acid pair mutants, when site II was native, with respect to the Ca$^{2+}$ association rate of the mutant with no site I acid pairs, I$_0$II$_X$. I$_0$II$_X$ had a calculated Ca$^{2+}$ association rate of $\sim 1.9 \times 10^7$ M$^{-1}$s$^{-1}$.
4.2.5 Ca\textsuperscript{2+} exchange rates of site II acid pair mutants with a functional Ca\textsuperscript{2+} binding site I

The above studies showed that the increase in Ca\textsuperscript{2+} affinity with increasing number of acid pairs was due primarily to an increase in the Ca\textsuperscript{2+} association rate. Our studies demonstrated that removal of the single X acid pair in site II produced a much greater decrease in Ca\textsuperscript{2+} affinity than deletion of the single Z acid pair in site I. Consistent with \(I_{2X}\) and \(W-I_{2X}\)'s low Ca\textsuperscript{2+} affinity their rates of Ca\textsuperscript{2+} dissociation were too rapid to be observed. Similarly, the Ca\textsuperscript{2+} dissociation rates for \(I_{2Y}\) and \(W-I_{2Y}\) were too rapid to observe with Quin-2 or Trp fluorescence. The Ca\textsuperscript{2+} dissociation rate for \(I_{2Y,Z}\) was too rapid to be observed with Quin-2 but occurred at 587 s\textsuperscript{-1} with \(W-I_{2Y,Z}\), following its decrease in Trp fluorescence. Thus, the Ca\textsuperscript{2+} dissociation rates from \(I_{2Y}\) and \(I_{2Y,Z}\) were too rapid to observe while their Ca\textsuperscript{2+} affinities were comparable to mutants whose Ca\textsuperscript{2+} dissociation rates could be observed. This implies that these mutations must produce a significant increase in the Ca\textsuperscript{2+} association rate. The Ca\textsuperscript{2+} dissociation rates for the other site II mutants are listed in table 4.2 (Quin-2 N-Term Off Rate and Tryptophan N-Term Off Rate). The introduction of a Y acid pair had little effect on slowing Ca\textsuperscript{2+} dissociation rate compared to the introduction of an X or Z acid pair in site II.
4.2.6 Mg\textsuperscript{2+} Binding to site I acid pair CaM mutants with a functional Ca\textsuperscript{2+}-binding site II

Malmendal \textit{et al.} (114) recently demonstrated by NMR that Ca\textsuperscript{2+}-binding site I of CaM bound Mg\textsuperscript{2+} with a Kd of ~1mM. We used the W-site I mutants, with site II unmodified, to determine what role specific acid pairs played in facilitating Mg\textsuperscript{2+} binding to site I. Figure 4.8 shows that W-I\textsubscript{Y}Z\textsubscript{L}X, W-I\textsubscript{Z}II\textsubscript{X}, W-I\textsubscript{I}III\textsubscript{X}, W-I\textsubscript{I}XX and W-I\textsubscript{II}III\textsubscript{X} bind Mg\textsuperscript{2+} and produce a Mg\textsuperscript{2+} dependent increase in Trp fluorescence. These proteins were half maximally saturated at pMg 3.4 for W-I\textsubscript{Y}Z\textsubscript{L}X, 3.1 for W-I\textsubscript{Z}II\textsubscript{X}, 1.8 for W-I\textsubscript{I}III\textsubscript{X}, 0.8 for W-I\textsubscript{I}II\textsubscript{X} and 0.7 for W-I\textsubscript{II}III\textsubscript{X}. When the native Z acid pair was removed from site I (W-I\textsubscript{II}III\textsubscript{X}), there was a dramatic ~240-fold decrease in Mg\textsuperscript{2+} affinity compared to W-I\textsubscript{Z}II\textsubscript{X}. Addition of a single X acid pair in site I (W-I\textsubscript{I}XX) had no effect on the low Mg\textsuperscript{2+} affinity observed in the site I acid pair knock out, while addition of the Y acid pair (W-I\textsubscript{II}III\textsubscript{X}) increased Mg affinity ~10-fold. Only the single Z acid pair in site I (W-I\textsubscript{II}III\textsubscript{X}) was able to support high Mg\textsuperscript{2+} affinity by producing an ~240-fold increase in Mg\textsuperscript{2+} affinity compared to W-I\textsubscript{II}III\textsubscript{X}. When the number of acid pairs was increased from one to two and then to three in site I, while leaving site II unmodified, only those mutants which contained a Z acid pair bound Mg\textsuperscript{2+} with a Kd ≥ 2mM Mg\textsuperscript{2+} (see table 4.3, Tryptophan N-Term Kd). Thus, high Mg\textsuperscript{2+} affinity required a Z acid pair in site I, unlike high Ca\textsuperscript{2+} affinity which was less selective and required only one acid pair located at any position in site I.
Figure 4.8: Mg$^{2+}$ binding to site I acid pair mutants with a functional site II.

The Figure shows the Mg$^{2+}$ dependent increase in Trp fluorescence for W-I$_{YZ}$II$_X$ (■), W-I$_2$II$_X$ (△), W-I$_Y$II$_X$ (○), W-I$_x$II$_X$ (□) and W-I$_o$II$_X$ (○) as a function of pMg. Increasing concentrations of Mg$^{2+}$ were added to 1 mL of each CaM (1 μM) in 200 mM MOPS, 90 mM KCl, 2 mM EGTA, pH 7.0 at 22°C to yield the indicated pMg. Tryptophan fluorescence was monitored at 335 nm with excitation at 275 nm. 100% fluorescence corresponds to a 1.4, 1.4, 1.4, 1.2 and 1.2-fold increase in tryptophan fluorescence for W-I$_{YZ}$II$_X$, W-I$_2$II$_X$, W-I$_Y$II$_X$, W-I$_x$II$_X$ and W-I$_o$II$_X$, respectively. Each data point represents an average of 3 titrations ± S.E. fit with a sigmoidal curve.
4.2.7 Mg$^{2+}$ exchange rates of site I acid pair mutants with a functional Ca$^{2+}$ binding site II

Figure 4.9 shows the EDTA induced decrease in Trp fluorescence which occurs upon Mg$^{2+}$ removal from the N-terminal Ca$^{2+}$-binding sites of the W-site I acid pair mutants. Mg$^{2+}$ dissociated from the N-terminal Ca$^{2+}$-binding sites of W-I$_{1_{Yz}}$II$_{X}$ at 257 s$^{-1}$, from W-I$_{2}$II$_{X}$ at 139 s$^{-1}$, from W-I$_{1_Y}$II$_{X}$ at 820 s$^{-1}$, from W-I$_{X}$II$_{X}$ at 222 s$^{-1}$ and from W-I$_{0}$II$_{X}$ at 224 s$^{-1}$ (see table 4.3, Tryptophan N-Term Off Rate). The histograms in Figure 4.10A compare the ratio of the Mg$^{2+}$ dissociation rates from each of the site I mutants to the Mg$^{2+}$ dissociation rate of W-I$_{0}$II$_{X}$. The Mg$^{2+}$ dissociation rates of the site I mutants either did not significantly change or were increased compared to W-I$_{0}$II$_{X}$. Since the increase in N-terminal Mg$^{2+}$ affinity observed with the increased number of acid pairs in the W-site I mutants cannot be explained by a slowing of the Mg$^{2+}$ dissociation rate, it must have occurred as a result of an increased Mg$^{2+}$ association rate. Figure 4.10B compares the ratio of the Mg$^{2+}$ association rates of all the W-site I mutants with respect to the Mg$^{2+}$ association rate of W-I$_{0}$II$_{X}$. The Mg$^{2+}$ association rate increased for all the W-site I mutants which exhibited an increase in Mg$^{2+}$ affinity compared to W-I$_{0}$II$_{X}$. Thus, an increase in the Mg$^{2+}$ association rate was responsible for the increase in Mg$^{2+}$ affinity of the W-site I mutants.
Figure 4.9: Mg\(^{2+}\) dissociation from W-site I acid pair mutants with a functional site II.

Figure 7A shows the decrease in tryptophan fluorescence associated with Mg\(^{2+}\) dissociation from the N-terminal sites of W-I\(_{1}\)II\(_{X}\), W-I\(_{2}\)II\(_{X}\), W-I\(_{1}\)I\(_{X}\), W-I\(_{1}\)II\(_{X}\), and W-I\(_{0}\)II\(_{X}\). Each CaM (14 \(\mu\)M) + Mg\(^{2+}\) (10 mM) + EGTA (500 \(\mu\)M) in 20 mM Hepes, pH 7.0 was rapidly mixed with an equal volume of EDTA (30 mM) in the same buffer at 10°C. CaM tryptophan fluorescence was monitored through a UV transmitting black glass filter (U-31 from Oriel, Stanford, CT) with excitation at 275 nm. Each trace is an average of at least 5 traces fit with a single exponential equation (variance <2.0x10\(^{-4}\)). Control experiments in which CaM + Mg\(^{2+}\) were reacted with buffer + Mg\(^{2+}\) were flat lines beginning at the starting amplitude of the EDTA traces. In another control, when 1mM Ca\(^{2+}\) was added to the CaM + Mg\(^{2+}\) mixtures and then mixed with EDTA, the rates observed were those obtained for Ca\(^{2+}\) dissociation as listed in table 2 (table 2, F19W Tryptophan N-Term Off Rate), implying we were originally observing truly Mg\(^{2+}\) dissociation and not Ca\(^{2+}\).
Table 4.3: Comparison of the Mg\(^{2+}\) affinities, dissociation rates and association rates of the W-site I acid pair mutants.

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Mutant Protein</th>
<th>Site I Acid Pairs</th>
<th>Site II Acid Pairs</th>
<th>Tryptophan N-Term Kd, Hill Coef</th>
<th>Tryptophan N-Term Off Rate (s(^{-1}))</th>
<th>Tryptophan N-Term On Rate (M(^{-1})s(^{1}))</th>
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<tr>
<td>W-(\Delta)II(_{xy})</td>
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<td>X</td>
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Figure 4.10: Comparison of the Mg$^{2+}$ dissociation and association rates for the W-site I acid pair mutants with a functional site II.

Figure 4.10A compares the ratio of Mg$^{2+}$ dissociation rates of all the W-site I acid pair mutants, when site II was native, with respect to the Mg$^{2+}$ dissociation rate of the mutant with no site I acid pairs, W-I$_0$II$_Z$. Figure 4.10B compares the ratio of Mg$^{2+}$ association rates of all the W-site I acid pair mutants, when site II was native, with respect to the Mg$^{2+}$ association rate of the mutant with no site I acid pairs, W-I$_0$II$_Z$. W-I$_0$II$_Z$ had Mg$^{2+}$ dissociation and association rates of $\sim$244/s and $1.3 \times 10^3$ M$^{-1}$s$^{-1}$, respectively.
4.2.8 Ca²⁺-dependent activation of phosphodiesterase by Ca²⁺-binding site I and II acid pair mutants

We have created a series of N-terminal mutants which displayed an ~560-fold range in Ca²⁺ affinities. These mutants allowed us to determine the effect that modified N-terminal Ca²⁺ affinity had on the Ca²⁺ dependent activation of PDE. Figure 4.11 shows the Ca²⁺-dependent activation of PDE by W-I₂II₂ (the highest affinity N-terminal CaM mutant, 2.21 μM Kd), I₂IIₓ (wtCaM), I₀IIₓ, I₂II₀, W-I₂IIₓ(no -z), W-I₀IIₓ(no -z), and W-I₀IIₓX(no -z) (the lowest affinity N-terminal CaM mutant, 1.24 mM Kd). Half-maximal activation of PDE for these proteins occurred at pCa 5.8 for W-I₂II₂, I₂IIₓ, and I₀IIₓ, at 5.7 for I₂II₀, 5.4 for W-I₂IIₓ(no -z), 5.2 for W-I₀IIₓ(no -z), and 5.3 for W-I₀IIₓX(no -z). The data shows that in the presence of a CaM-binding target enzyme, mutants which exhibited a 560-fold range in N-terminal Ca²⁺ affinity exhibited only a 2.9-fold range in their Ca²⁺ dependent activation of PDE.

Elimination of the Z acid pair in site I (I₀IIₓ) or the X acid pair in site II (I₀II₀) produced a 3.7 and 27-fold decrease in N-terminal Ca²⁺ affinity, yet both mutants show a similar Ca²⁺ dependent activation of PDE as wtCaM (I₂IIₓ).

Elimination of Ca²⁺ binding to site I or site II by mutation of the -z Glu to Ala (W-I₀IIₓ(no -z), W-I₂IIₓ(no -z), respectively) produced even larger decreases in N-terminal Ca²⁺ affinity (96 and 59-fold relative to wtCaM, respectively) and these mutants half-maximally activated PDE at pCa 5.2 and 5.4 respectively. Even the lowest affinity N-terminal mutant (W-I₀IIₓX(no -z)) which had 133-fold lower N-terminal Ca²⁺ affinity
than wtCaM half-maximally activated PDE at pCa 5.3. These results suggest that
the decreases in Ca\(^{2+}\) affinity that occurred upon elimination of the acid pairs in site I
and II could be almost completely compensated for upon target enzyme binding.
Figure 4.11: Ca\textsuperscript{2+} dependent activation of Phosphodiesterase by site I and site II acid pair mutants.

The figure shows the Ca\textsuperscript{2+} dependent activation of a CaM-dependent phosphodiesterase activated by W-I\textsubscript{2II} (●), I\textsubscript{2II} (■), I\textsubscript{OII} (▲), I\textsubscript{2I0} (△), W-I\textsubscript{2II} (□), W-I\textsubscript{0II} (○), W-I\textsubscript{OII} (□) and W-I\textsubscript{0II} (Δ). Increasing concentrations of Ca\textsuperscript{2+} were added to 1 mL of each CaM (100 - 300 nM) in 200 mM MOPS, 90 mM KCl, 2 mM EGTA, 3 mM Mg\textsuperscript{2+}, 8 μM Mant-cGMP, 15 μM phosphodiesterase, pH 7.0 at 22°C to yield the indicated pCa. The data were obtained by fitting the initial linear phase of the hydrolysis of Mant-cGMP, with 100% activity corresponding to that reached by wtCaM at pCa 4.0. Each data point represents an average of 3 titrations ± S.E. fit with a sigmoidal curve.
4.3 Discussion

Our studies were designed to test the acid pair hypothesis in the N-terminal Ca\(^{2+}\)-binding sites of CaM. When the -Z Glu in CaM's site II was mutated to Ala we observed an \(\sim\)120-fold reduction in N-terminal Ca\(^{2+}\) affinity compared to the corresponding wt construct (W-I\(_2\)II\(_2\)). When we removed the endogenous Z acid pair in site I, in the presence of impaired Ca\(^{2+}\) binding to site II (W-I\(_0\)II\(_2\)(Z)), N-terminal Ca\(^{2+}\) affinity decreased another \(\sim\)2-fold. Utilizing this mutant, we then increased the number of acid pairs in site I from zero to one, to two and to three. The introduction of any one or two acid pairs into site I increased Ca\(^{2+}\) affinity only \(\sim\)2-fold, while the introduction of three acid pairs into site I produced a dramatic \(\sim\)40-fold increase in N-terminal Ca\(^{2+}\) affinity compared to the no acid pair mutant.

Consistent with our results, Haiech et al. (126) also generated an -Z Glu to Ala site II CaM mutant in which N-terminal Ca\(^{2+}\) affinity was decreased \(\sim\)300-fold. Ca\(^{2+}\)-binding site III of CaM has three acids but no acid pairs and Wu and Reid (62) have tested the acid pair hypothesis in this EF-hand of CaM. They mutated the +Z Asp to Glu in site IV decreasing Ca\(^{2+}\) affinity to this site by \(\sim\)3000-fold (127). This mutation also reduced Ca\(^{2+}\) affinity to site III by \(\sim\)24-fold compared to their wtCaM construct.

Upon incorporating an X or Z acid pair in site III with an impaired site IV, Wu and Reid observed dramatic \(\sim\)58 and 110-fold increases in site III's Ca\(^{2+}\) affinity, respectively. Thus, introducing an X or Z acid pair into site I of CaM produces a much smaller increase in N-terminal Ca\(^{2+}\) affinity than does introduction of an X or Z acid into site III of CaM, when the partner EF-hand was impaired. This could be due to the more rigid structure observed for the N-terminal domain of CaM compared to its more flexible C-terminal domain (128). The increased flexibility of the C-terminal domain may allow modifications in the Ca\(^{2+}\)-binding loop domains to
play a larger role in determining Ca\(^{2+}\) affinity.

When we increased the number of acid pairs in site I from one to two, we observed little change in Ca\(^{2+}\) affinity when site II was impaired. Similarly, Wu and Reid observed only an ~ 2-fold increase in site III Ca\(^{2+}\) affinity upon increasing the number of acid pairs from 1 (X acid pair) to two (X and Z acid pair) in their site IV impaired mutant (62). When we increased the number of acid pairs to three in site I, this mutant placed five negatively charged residues in site I's Ca\(^{2+}\) coordination shell. We observed a dramatic ~17-fold increase in Ca\(^{2+}\) affinity compared to the single and double acid pair mutants. The increase in Ca\(^{2+}\) affinity that we observe with three acid pairs in site I is contrary to the acid pair hypothesis which states that Ca\(^{2+}\) affinity will be maximized when four acids are paired on the X and Z axes (62).

Introduction of a fifth ligating carboxylate was shown to decrease cation affinity ~2 to 8-fold in a synthetic EF-hand peptide corresponding to site III of TnC, presumably due to increased interligand charge repulsion (129). In support of our findings, introduction of a fifth carboxylate ligand in either the CD or EF Ca\(^{2+}\)-binding sites of oncomodulin, produced an ~10-fold increase in Ca\(^{2+}\) affinity for both the individual CD and EF Ca\(^{2+}\)-binding site mutants (64). The general conclusion from our studies of the site I mutants with an impaired site II is that increasing the number of acid pairs in site I of CaM caused little increase in Ca\(^{2+}\) affinity, until all the acid pairs were filled.

Removal of the single X acid pair in site II, when site I was native, produced a much more dramatic 27-fold decrease in Ca\(^{2+}\) affinity. The subsequent introduction of an X, or Z acid pair in site II produced an ~30-fold increase in Ca\(^{2+}\) affinity, while introduction of an Y acid pair increased Ca\(^{2+}\) affinity ~10-fold. This high Ca\(^{2+}\) affinity only requires a single X or Z acid pair in site II since further additions of any two or three acid pairs in site II, caused little further increase in Ca\(^{2+}\) affinity, when site I was native.
Removal of the single Z acid pair in site I produced only an ∼4-fold decrease in Ca\(^{2+}\) affinity, when site II was native. Introduction of any single acid pair to site I could restore this loss in Ca\(^{2+}\) affinity. The larger increase in Ca\(^{2+}\) affinity caused by increasing the number of acid pairs in site II compared to site I must reflect structural differences between the two sites. One striking difference between the two sites is that the helices of site I are more hydrophobic than the corresponding helices in site II. Helix 1 of site I is 1.4 times more hydrophobic than helix 3 of site II (hydrophobic index = -5.44 and -7.7 kcal/mol, respectively); similarly helix 2 of site I is ∼12 times more hydrophobic than helix 4 of site II (-0.77 and -9.09 kcal/mol, respectively). The larger hydrophobicity of site I may make this site more stable and thus more rigid than site II. The greater flexibility of site II compared to site I may allow changes in the loop, such as increased number of acid pairs, to have a larger effect on Ca\(^{2+}\) affinity. It may be that the helices in site I play a predominant role in maintaining high N-terminal Ca\(^{2+}\) affinity. Consistent with this idea, we have recently shown that increasing the hydrophobic content of the flanking helices in site III of CaM can increase site III’s Ca\(^{2+}\) affinity (63).

Furthermore, site I contains only a single Met residue in helix 2, whereas site II contains one Met residue in helix 3 and three Met residues in helix 4. Due to the high flexibility and greater solubility of Met residues over the other hydrophobic residues (130, 131), these Met residues may further increase the flexibility of site II and decrease the energy needed to expose these residues to solvent upon Ca\(^{2+}\) binding (128). Thus, the acid pairs in site II play a much greater role in determining N-terminal Ca\(^{2+}\) affinity than the acid pairs in site I. Consistent with this, Maune et al. (58) have shown that -Z Glu to Lys or Gln mutations in site II are more deleterious to N-terminal Ca\(^{2+}\) affinity than the corresponding mutations in site I.
Addition of a single X or Z acid pair in site II increased the number of charged residues in site II to four. Both of these single acid pairs increased N-terminal Ca\textsuperscript{2+} affinity to a similar extent. Consistent with our results, it has been shown in synthetic EF-hands, and in a whole protein, that when there are four acidic residues in a Ca\textsuperscript{2+}-binding loop, that the single X or Z acid pairs increase Ca\textsuperscript{2+} affinity similarly (62, 72). The Z acid pair has been proposed to increase Ca\textsuperscript{2+} affinity by being the first set of chelating residues to initiate Ca\textsuperscript{2+} coordination (72). Consistent with this, the \(~30\)-fold increase in Ca\textsuperscript{2+} affinity upon incorporation of an Z acid pair in site II was reflected by its \(~15\)-fold faster Ca\textsuperscript{2+} on-rate and by its \(~2\)-fold slower Ca\textsuperscript{2+} dissociation rate, compared to the no acid pair mutant. The X acid pair may increase Ca\textsuperscript{2+} affinity with the aid of the other two acid residues (+Y Asp and -Z Glu) since both the -X and +Y Asp residues stabilize (through hydrogen bonds) the water molecule that coordinates the Ca\textsuperscript{2+} ion in site II (132). The \(~27\)-fold increase in Ca\textsuperscript{2+} affinity upon incorporation of an X acid pair in site II was reflected by its \(~10\)-fold faster Ca\textsuperscript{2+} on-rate and by its \(~3\)-fold slower Ca\textsuperscript{2+} dissociation rate, compared to the no acid pair mutant.

The gateway hypothesis proposes that an EF-hand that contains an Asp residue at the -X position should have a slower cation dissociation rate than one that has a neutral sidechain (68). Consistent with this aspect of the gateway hypothesis, when the -X Asp in site II was mutated to Asn (I\(_{II}I_{0}\)) the Ca\textsuperscript{2+} dissociation rate from the N-terminal Ca\textsuperscript{2+}-binding sites of CaM increased \(~3\)-fold compared to wtCaM. Furthermore, all site II mutants which contained an X acid pair, with a native site I, had slower Ca\textsuperscript{2+} dissociation rates than the no acid pair mutant. The -X residue was not the only residue to affect Ca\textsuperscript{2+} dissociation in the site II mutants. Since incorporation of an Z acid pair decreased the Ca\textsuperscript{2+} dissociation rate to a similar extent as the X acid pair our results are more consistent with the X or Z acid pair's
electro-static attraction being responsible for the Ca\textsuperscript{2+} dissociation than the gateway hypothesis. Furthermore, Falke and colleagues observed that incorporation of an Asp at the gateway residue decreased Ca\textsuperscript{2+} affinity ~16-fold in the galactose binding protein which contains a single EF-hand like Ca\textsuperscript{2+}-binding site (133). This is contrary to the ~30-fold increase in Ca\textsuperscript{2+} affinity that we observed upon addition of Asp at the gateway residue (-X) in the site II mutants of CaM. It could be that complex interactions between the multiple ligating residues in the two proteins are quite different giving rise to the differential effects of incorporation of the -X Asp residue on Ca\textsuperscript{2+} affinity.

Incorporation of the single Y acid pair into site II produced only an ~10-fold increase in Ca\textsuperscript{2+} affinity. Thus, an Y acid pair is less effective than an X or Z acid pair at increasing N-terminal Ca\textsuperscript{2+} affinity. The reason for this is that the Y acid pair increased the Ca\textsuperscript{2+} dissociation rate ~2-fold while increasing the Ca\textsuperscript{2+} association rate ~20-fold, compared to the no acid pair mutant. Thus, the Y acid pair increases both the Ca\textsuperscript{2+} dissociation rate and the Ca\textsuperscript{2+} association rate to the N-terminal of CaM when placed in site II. NMR solution structures of CaM show that upon Ca\textsuperscript{2+} binding Thr 62 (-Y position of site II) becomes more buried and less solvent exposed (27, 134). When this residue is mutated to Asp, and if the same Ca\textsuperscript{2+}-induced conformational changes occur to the mutated residue as occur to the native Thr residue, then T62D should destabilize the Ca\textsuperscript{2+} bound state of the N-terminal of CaM. This may explain why the T62D mutation increases the Ca\textsuperscript{2+} dissociation rate and produces smaller increases in Ca\textsuperscript{2+} affinity. Consistent with this interpretation, Kragelund et al. (22) demonstrated that hydrophobic core mutations throughout calbindin D\textsubscript{9k} that caused a destabilization of the apo protein and a stabilization of the Ca\textsuperscript{2+}-loaded protein caused a correlative increase in Ca\textsuperscript{2+} affinity for the mutant protein by slowing the Ca\textsuperscript{2+} dissociation rate. Furthermore, when TnC binds Ca\textsuperscript{2+}
Met 82 becomes more exposed to solvent (135). When this residue was mutated to the more polar Gln, Ca\(^{2+}\) affinity was increased ~4-fold reflected by an ~4-fold slowing of the Ca\(^{2+}\) dissociation rate (135, 136). These studies showed that exposure of a more polar residue to solvent upon Ca\(^{2+}\) binding can increase Ca\(^{2+}\) affinity by slowing the rate of Ca\(^{2+}\) dissociation.

Our current data indicates that an increase in the Ca\(^{2+}\) association rate was the main contributing factor for the observed increase in Ca\(^{2+}\) affinity upon increasing the number of acid pairs in Ca\(^{2+}\)-binding site II. It could be that the introduction of the negative charged residues causes an increase the Ca\(^{2+}\) association rate, first by an increased electrostatic attraction and, perhaps secondly by aiding in the removing of the water shell from the Ca\(^{2+}\) ion. It is generally thought that the rate limiting step of Ca\(^{2+}\) binding to a protein is removal of the water shell from the Ca\(^{2+}\) ion (9).

Consistent with our results, Martin et al. (66) have shown that reducing 3 negative surface charges near the Ca\(^{2+}\)-binding domain in calbindin D\(_{9k}\) reduced the Ca\(^{2+}\) association rate ~50-fold. Furthermore we have shown that CaM's C-terminal Ca\(^{2+}\) affinity could be increased ~3-fold when the chelating residues in Ca\(^{2+}\)-binding site III of cardiac TnC were substituted into CaM's Ca\(^{2+}\)-binding site III (63). This increased the number of acid pairs in CaM's site III from zero to two by adding an X and Z acid pair. The increase in C-terminal Ca\(^{2+}\) affinity of this CaM mutant was almost exclusively due to an ~3-fold increase in the Ca\(^{2+}\) association rate. Thus, increasing the number of acid pairs in an EF-hand can produce an increase in the rate of Ca\(^{2+}\) binding.

Incorporation of two acid pairs in site II tend to have slightly faster Ca\(^{2+}\) association rates than the single acid pair mutants. Besides aiding in dehydration of the Ca\(^{2+}\) ion, incorporation of multiple acid pairs may cause the binding pocket of the EF-hand to be expanded due to charge repulsion of the ligating residues (9, 63,
This larger pocket may allow Ca$^{2+}$ to associate with the EF-hand faster due to less steric hindrance of Ca$^{2+}$ binding.

Our studies with site I mutants showed small (3 to 4-fold) increases in Ca$^{2+}$ affinity with the introduction of an X, Y or Z acid pair in site I when site II was native. Thus, the addition of a single acid pair in site I when site II was native or impaired (no-Z mutant) produced similar small increases in Ca$^{2+}$ affinity. Interestingly, the increase in Ca$^{2+}$ affinity observed with incorporation of the single X acid pair in site I with a native site II was due solely to an ~4-fold slowing of the Ca$^{2+}$ dissociation rate. Incorporation of an Z or Y acid pair in site I had little effect on Ca$^{2+}$ dissociation rate, thus they increased Ca$^{2+}$ affinity by increasing the Ca$^{2+}$ association rate. Thus, in site I only the X acid pair is able to drastically slow the Ca$^{2+}$ dissociation rate. This is consistent with the gateway hypothesis, except that the X acid pair causes an increase in Ca$^{2+}$ affinity and not a decrease.

Increasing the number of acid pairs in site I, from one to two by adding an Y acid pair in site I to an X or Z acid pair slightly increased N-terminal Ca$^{2+}$ affinity, while addition of an X acid pair to the Z acid pair had little effect on Ca$^{2+}$ affinity, when site II was native. Further incorporation of three acid pairs in site I, when site II was native, had little effect on N-terminal Ca$^{2+}$ affinity compared to the double acid pair mutants. In general the small increase in Ca$^{2+}$ affinity observed with the site I mutants, when site II was native, was due primarily to an ~3 to 4-fold increase in the Ca$^{2+}$ association rate, except for the single X acid pair mutant.

The fact that we did not observe as large of an increase in N-terminal Ca$^{2+}$ affinity with addition of three acid pairs in site I, when site II was native compared to when site II was impaired, may be due to an increase in the electrostatic repulsion of the chelating residues. This effect may be the reason for $I_{X,Y,Z}II'x$'s slightly faster Ca$^{2+}$ dissociation and association rates compared to most of the other site I acid pair
mutants, possibly due to a larger Ca\(^{2+}\)-binding pocket. This charge repulsion of the three acid pairs may be compensated for by a more flexible N-terminal domain when the \(-Z\) Glu was mutated to Ala in site II. Recent NMR structures of EF-hand proteins with a \(-Z\) Glu residue mutated to Ala or Gln in one of the paired Ca\(^{2+}\)-binding sites shows that the Ca\(^{2+}\) bound structure of the domain which contains the mutation was more flexible than the Ca\(^{2+}\)-bound structure of the native protein (140, 141).

Recently both Ohki et al. (116) and Malmendal et al. (114) have shown by high resolution NMR that CaM’s N-terminal Ca\(^{2+}\)-binding site I binds Mg\(^{2+}\) with a physiologically relevant Kd of ~1mM. Unlike Ca\(^{2+}\) binding, Mg\(^{2+}\) binding to site I caused only local structural changes (116). Furthermore, the Mg\(^{2+}\) induced local structural changes in site I were distinct to the N-terminal portion of the first EF-hand, since there were little to no resonance changes in the last three residues in the Ca\(^{2+}\) binding loop (116). This may imply that the potential Mg\(^{2+}\) ligating residues in CaM’s site I are located at the \(+X\), \(+Y\), \(+Z\) and \(-Y\) positions (116). Our current data suggests that Ca\(^{2+}\)-binding site I of CaM can bind Mg\(^{2+}\) with a Kd of ~0.8mM, consistent with the NMR data. Procysyn and Reid (72, 73) showed that only synthetic EF-hands which had a Z acid pair bound Mg\(^{2+}\). We wanted to test this finding using our acid pair mutants in Ca\(^{2+}\)-binding site I of CaM.

In the absence of the \(+Z\) coordinating Asp 24 (mutated to Asn in W-Io\(II_X\)) we observed an ~240-fold reduction in Mg\(^{2+}\) affinity. Furthermore, after removal of the endogenous Z acid pair in site I, Mg\(^{2+}\) affinity was not affected by introduction of an X acid pair (W-Ix\(II_X\)) and was only increased ~10-fold upon introduction of a Y acid pair (W-Iy\(II_X\)). Maximal Mg\(^{2+}\) binding (as well as maximal Ca\(^{2+}\) binding) was
observed with two acid pairs located at the Y and Z axes (W-I_{YZ}II_{X}) which exhibited
~1.8-fold greater Mg\(^{2+}\) affinity than the Z acid pair alone (W-I_{Z}II_{X}). Thus, Ca\(^{2+}\)-
binding site I mutants that lacked a Z acid pair showed drastic reductions in Mg\(^{2+}\)
binding. This implies that an Z acid pair was required for high Mg\(^{2+}\) affinity in Ca\(^{2+}\)-
binding site I of CaM, consistent with the synthetic EF-hand peptide studies of Reid
and colleagues (71, 72).

Reid \textit{et al.} (71, 72) have suggested that Mg\(^{2+}\) affinity could be increased
without increasing Ca\(^{2+}\) affinity from their studies of synthetic EF-hand peptides.
Consistent with this we find that addition of an Z acid pair to site I was more
affective at increasing the Mg\(^{2+}\) affinity (~240-fold increase) than it was at increasing
the Ca\(^{2+}\) affinity (~4-fold increase). Furthermore, addition of a Z acid pair to any
single or double acid pairs in site I always increased Mg\(^{2+}\) affinity to a greater extent
than it increased Ca\(^{2+}\) affinity. Similarly, when a Z acid pair was added to an X acid
pair in the CD Ca\(^{2+}\)-binding site of oncomodulin, Mg\(^{2+}\) affinity increased ~50-fold,
whereas Ca\(^{2+}\) affinity was only increased ~10-fold (64). Thus, addition of an Z acid
pair in site I of CaM and the CD site of oncomodulin serves to selectively increase
Mg\(^{2+}\) affinity more than Ca\(^{2+}\) affinity. It remains to be determined if this
generalization applies to other EF-hands.

We observed Mg\(^{2+}\) dissociation from site I of W-I_{Z}II_{X} (W-wtCaM) at 139 s\(^{-1}\)
at 10\(^{\circ}\)C and assuming a Q10 of 2 (137) we calculated a Mg\(^{2+}\) off-rate of ~380/s at
25\(^{\circ}\)C. This is the rate observed for Mg\(^{2+}\) dissociation from CaM's site I in the NMR
studies of Malmendal \textit{et al.} (114). The general trend observed for Mg\(^{2+}\) dissociation
was that an Z acid pair slowed, an Y acid pair increased and an X acid pair had no
effect on the Mg$^{2+}$ dissociation rates. Mg$^{2+}$ dissociation was not affected by the
gateway residue, thus Mg$^{2+}$ must use other factors to govern its dissociation rates
from the N-terminal site I of CaM. The Z acid pair produced a large ~140-fold
increase in the Mg$^{2+}$ association rate while an Y acid pair produced only an ~40-fold
increase and an X acid pair did not affect the Mg$^{2+}$ association rate. Thus, the Z acid
pair increases Mg$^{2+}$ affinity not only by slowing Mg$^{2+}$ dissociation but also by
increasing the Mg$^{2+}$ association rate.

Addition of an X acid pair to an Y acid pair had no effect of the Mg$^{2+}$ affinity,
dissociation or association rates when compared to the single Y acid pair mutant.
Addition of an Y acid pair to an Z acid pair increased the Mg$^{2+}$ dissociation rate
~2.3-fold, while increasing the Mg$^{2+}$ association rate ~4.3-fold, consistent with this
mutant’s ~2-fold higher Mg$^{2+}$ affinity compared to the single Z acid pair mutant.
When an X acid pair was added to the Y and Z double acid pair mutant, the Mg$^{2+}$
dissociation rate increased ~1.9-fold and the Mg$^{2+}$ association rate only increased
~1.4-fold decreasing the Mg$^{2+}$ affinity ~1.4-fold.

The binding of CaM to target peptides and proteins produce dramatic
increases in CaM’s affinity for Ca$^{2+}$ by decreasing CaM’s N-terminal and C-terminal
Ca$^{2+}$ dissociation rates ~200 and 20-fold, respectively (43, 138). It was surprising to
find that the two mutants which displayed the largest (~560-fold) difference in N-
terminal Ca$^{2+}$ affinity only exhibited ~4-fold differences in their Ca$^{2+}$-dependent
activation of PDE.. Furthermore, the loss of the X acid pair in site II caused ~30-
fold loss in Ca^{2+} affinity, but exhibited identical Ca^{2+}-dependent activation of PDE as wtCaM. Clearly the large loss in N-terminal Ca^{2+} affinity in mutants like \( \text{I}_{0}\text{II}_{X(\text{in}-2)} \) can be compensated for by the presence of the CaM-binding enzyme. Consistent with our findings, Haiech et al. (127) demonstrated that in the presence of a CaM-binding peptide the Ca^{2+}-binding properties of mutant CaMs similar to our mutant W-I_{2}\text{II}_{X(\text{in}-2)} \) could be restored to those of the wtCaM. Wu and Reid (126) recently generated a CaM mutant which exhibited 25-fold and 2760-fold loss in Ca^{2+} affinity to sites III and IV, respectively, by mutating the +X Asp to Glu in site IV. This mutant showed impaired Ca^{2+}-dependent activation of PDE at low [Ca^{2+}]. These results suggest that altering C-terminal Ca^{2+} affinity may have more effect on the Ca^{2+}-dependent activation of PDE than altering N-terminal Ca^{2+} affinity.

Maune et al. (58) generated a series of CaM mutants that had up to ~16-fold decreased N- and C-terminal Ca^{2+} affinities compared to wtCaM by replacing the -Z chelating residue in each of the four Ca^{2+} binding sites individually with Gln or Lys. Some of these mutants shifted the Ca^{2+}-dependent activation of skeletal muscle myosin light chain kinase and Ca^{2+}-ATPase over an ~100-fold range (139). These studies showed that low Ca^{2+} affinity C-terminal CaM mutants required more Ca^{2+} to activate Ca^{2+}-ATPase than low affinity N-terminal mutants, whereas the both low Ca^{2+} affinity N-terminal and C-terminal CaM mutants displayed right shifted Ca^{2+}-dependent activation of skeletal muscle MLCK (139).
Our Glu to Ala mutants at the -Z position in both site I and II exhibited greater than ~120-fold decreases in N-terminal Ca\(^{2+}\) affinity and only ~4-fold lower Ca\(^{2+}\) dependent activation of PDE. The no -Z mutants exhibited a slight decrease in \(V_{\text{Max}}\). One explanation for the decreased \(V_{\text{Max}}\) and right shifted Ca\(^{2+}\)-dependent activation of PDE caused by the site I and II (no -Z) mutants may be due to a reduced ability of these mutants to exhibit a Ca\(^{2+}\)-dependent exposure of their N-terminal hydrophobic pockets. Recent NMR studies have shown that when the -Z Glu residue in Ca\(^{2+}\)-binding site I of troponin C or Ca\(^{2+}\)-binding site III in CaM was mutated to Ala, these proteins could not expose, or exposed less, of their respective domain’s hydrophobic pockets (140, 141). Thus, altering the Ca\(^{2+}\) affinity of the N-terminal of CaM has less of an effect on the Ca\(^{2+}\)-dependent activation of PDE.

We have shown that incorporation of a single acid pair in either N-terminal Ca\(^{2+}\)-binding sites of CaM can increase Ca\(^{2+}\) affinity for these mutants, while only the single Z acid pair in site I was able to support high Mg\(^{2+}\) affinity. The increase in Ca\(^{2+}\) affinity is consistent with the acid pair hypothesis, although maximal Ca\(^{2+}\) affinity was not achieved with the X and Z acid pair. The acid pair induced increases in Ca\(^{2+}\) affinity were much greater in site II than they were in site I. Increasing the number of acid pairs to two and then to three in sites I and II, when the other site was native, had little effect on Ca\(^{2+}\) affinity. The observed increases in both Ca\(^{2+}\) and Mg\(^{2+}\) affinities were shown to be primarily due to an increase in the cation association rates, except for the single X acid pair mutant in site I (I\(_X\)II\(_X\)) which only exhibited a reduced Ca\(^{2+}\) dissociation rate consistent with the gateway hypothesis.
Finally, the binding of a CaM target enzyme restored the Ca\(^{2+}\)-binding properties of low Ca\(^{2+}\) affinity N-terminal CaM mutants, since these mutants exhibited nearly wtCaM Ca\(^{2+}\)-dependent activation of the enzyme.

4.4 Conclusion

1. Increasing the number of acid pairs from zero to one and from one to two in site I, while site II was impaired, had little effect on N-terminal Ca\(^{2+}\) affinity while incorporation of three acid pairs dramatically increased Ca\(^{2+}\) affinity ~ 17-fold.

2. Increasing the number of acid pairs from zero to one at any position in site I, with a native site II, increased N-terminal Ca\(^{2+}\) affinity ~ 4-fold, while further addition of two or three acid pairs had little effect on Ca\(^{2+}\) affinity.

3. Addition of the single Z or X acid pair in site II, with a native site I, exhibited a large increase in N-terminal Ca\(^{2+}\) affinity ~ 30-fold, while further addition of two or three acid pairs had little effect on Ca\(^{2+}\) affinity.

4. High Mg\(^{2+}\) affinity at site I required a Z acid pair.

5. Both the increase in Ca\(^{2+}\) and Mg\(^{2+}\) affinity were primarily due to an increase in the cation association rates, except for the single X acid pair in site I, where the decrease in the dissociation rate explained the increase in Ca\(^{2+}\) affinity.

6. An X acid pair had more of an effect on decreasing the Ca\(^{2+}\) dissociation rate than an Y or Z acid pair in site I and than an Y acid pair in site II.

7. These acid pair mutants exhibited ~560-fold range in N-terminal Ca\(^{2+}\) affinities, but only expressed an ~ 4-fold range in Ca\(^{2+}\)-dependent activation of PDE.
4.5 Future Experiments

With the use of site-directed mutagenesis it is possible to explore the role that position and number of acid pairs play in other EF-hand systems. One could decrease or increase the number of acid pairs in an EF-hand using a similar approach as taken in this chapter. The obvious first choice would be Ca\(^{2+}\)-binidng sites III and IV in the C-terminal of CaM (leaving one of the sites native). One could also modify parvalbumin, oncomodulin, TnC and calbindin, just to name a few of the well characterized Ca\(^{2+}\)-binding proteins. This would determine, in different EF-hand proteins, the role that acid pairs play in maintaining or modifying Ca\(^{2+}\) affinity.

Upon completion of the C-terminal mutations in CaM, there should then be a series of CaM's created that have a broad range of N- and C-terminal Ca\(^{2+}\) affinities. These mutants would allow one to look at the Ca\(^{2+}\)-dependent activation of any number of CaM dependent enzymes, such as smooth muscle MLCK. This would determine if the binding of a particular enzyme could correct for the loss of Ca\(^{2+}\) affinity to the mutated domain. One could also determine, when differences are observed, the kinetics of the Ca\(^{2+}\)-CaM-target enzyme/peptide interactions using various stopped-flow fluorescence techniques, possibly utilizing artificial Ca\(^{2+}\) transients.

These CaM mutants could also be used to probe a CaM-dependent physiological response, such as smooth muscle contraction. Utilizing CaM mutants that were observed to have varying Ca\(^{2+}\)-dependent activation profiles of MLCK or altered Ca\(^{2+}\)-binindg kinetics, such as faster Ca\(^{2+}\) dissociation rates in the presence of
the enzyme compared to wtCaM will allow one to probe the contraction/relaxation cycle of smooth muscle by CaM. One might be able to address if the binding or dissociation of Ca$^{2+}$-CaM to MLCK is the rate-limiting step of smooth muscle contraction or relaxation, respectively.

With respect to the N-terminal CaM mutants one could begin to address the role that the flexibility of site I versus site II plays in determining N-terminal Ca$^{2+}$ affinity. First, the Met residues in site II could be mutated to more hydrophobic amino acids, such as Leu, either together or individually. These mutations could be done in the presence and absence of different site II acid pair mutations to see if modifying the acid pairs in site II still plays a large role in N-terminal Ca$^{2+}$ affinity in the presence of a less flexible (more hydrophobic) site II mutation. I would predict that when all the Met residues are made Leu (assuming that these mutations make site II less flexible) that modification of the acid pairs in site II will not have as big an effect on N-terminal Ca$^{2+}$ affinity as they do with the Met residues present. The reverse experiments could be performed in site I by replacing the corresponding hydrophobic residues with Met residues, and then modifying the number and location of acid pairs in site I.

One could also exchange the whole Ca$^{2+}$-binding loop domains of site I and site II, by placing them in the environment of the other loop. This would place the loop of site II in the hydrophobic environment of site I and visa versa. One could then modify the number and location of acid pairs in these sites, while leaving the other site unmodified. If the flexibility/hydrophobicity of site I is the key feature of
determining N-terminal Ca\(^{2+}\) affinity for this site, then the set of site II mutant exchanges should not have as big as an effect at modifying N-terminal Ca\(^{2+}\) affinity in this environment as they did in the site II environment. One might expect the opposite to be true of the site I loop exchange mutants with site II’s loop. If acid pairs in site II are playing a key role in determining N-terminal Ca\(^{2+}\) affinity then the site I exchange acid pair mutants should have a larger effect at modifying N-terminal Ca\(^{2+}\) affinity than they did in their native site I environments. If these predictions do not occur then it might be that non-chelating residues in each loop domain are playing a key role in determining N-terminal Ca\(^{2+}\) affinity for these sites. It might then be necessary to begin to modify (in the native loop I and II sites) the hydrophobicity, charge or length of non-chelating residues in the two loops to determine the effect of these types of mutations on N-terminal Ca\(^{2+}\) affinity.
APPENDIX

COMPUTER MODELING OF THE TRANSIENT OCCUPANCY OF TROPONIN C AND THE N-TERMINAL OF CALMODULIN BINDING TO MYOSIN LIGHT CHAIN KINASE

5.1 Computer Modeling of the Transient Occupancy of TnC

Figure 3.4 uses the changes in TnC-danz fluorescence to show the time course of Ca$^{2+}$ binding and dissociation from the N-terminal Ca$^{2+}$-specific sites of TnC during ACTs of increasing duration. Figure 5.1 shows the computer modeling of the time course of Ca$^{2+}$ binding and dissociation from the N-terminal Ca$^{2+}$-specific sites of TnC during ACTs of increasing duration. We used a computer simulation which fixed the Ca$^{2+}$ off-rate from TnC-danz at 160/s (Ca$^{2+}$ off-rate at 10°C, see table 3.1) and the Ca$^{2+}$ on-rate to the Ca$^{2+}$-specific regulatory sites of TnC-danz at \( \approx 8.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1} \) at 10°C to model the experimental data. When 100 μM Ca$^{2+}$ was mixed with 1 μM TnC-danz in the presence of 0.375 mM EGTA (after mixing concentrations) (Figure 5.1, 0.75 mM EGTA trace) a 1.6 ms hw ACT was generated and Ca$^{2+}$ initially bound and transiently occupied \( \approx 100\% \) of the Ca$^{2+}$-specific regulatory sites of TnC-danz. As Ca$^{2+}$ was subsequently chelated by EGTA and removed from TnC-danz, the Ca$^{2+}$ dissociated from TnC-danz back to its Ca$^{2+}$-free level at a rate of \( \approx 100/\text{s} \). As [EGTA] was successively increased from 0.375 mM to
2.5, and 5 mM EGTA (after mixing concentrations) (Figure 5.1) ACTs of 0.21 and 0.1 ms hws were created and Ca\(^{2+}\) transiently occupied ~75 and 50% of the N-terminal regulatory sites of TnC-danz, respectively. Furthermore, as [EGTA] was increased from 0.375 to 2.5 and 5 mM EGTA, Ca\(^{2+}\) was chelated and removed from TnC-danz more quickly at rates of 155 and 160/s, respectively. As can be seen from the modeling, these simulations approximate the data quite well and confirm the predicted rates of Ca\(^{2+}\) association to the N-terminal regulatory sites of TnC-danz.
Figure 5.1: Computer simulation of the Ca$^{2+}$ occupancy of the N-terminal Ca$^{2+}$ specific regulatory sites of troponin C during artificial Ca$^{2+}$ transients of different duration.

The traces show computer simulations (using KSIM) of the time courses of transient Ca$^{2+}$ binding to TnC-danz when 100 µM Ca$^{2+}$ was mixed with 1 µM TnC-danz in the presence of 0.375, 2.5, or 5 mM EGTA, at 10°C. At time = 0, the initial [Ca$^{2+}$], [EGTA] and [TnC-danz] were set to the concentrations representing the amount of species that would be present in the stopped-flow apparatus just after mixing was complete (assuming infinitely fast mixing time). The simulation with no EGTA present (No EGTA trace) in which TnC-danz was mixed with 100 µM Ca$^{2+}$, showed that nearly 100% of TnC-danz bound Ca$^{2+}$. Ca$^{2+}$ on and off-rates from EGTA and TnC-danz were used as listed in table 3.1.
5.2 Computer Modeling of the N-Terminal of CaM Binding to MLCK Following ACTs of Various Duration

Wheat CaM-MIANS exhibits a large fluorescence increase which is specific for its binding to target proteins including MLCK (74). This fluorescent CaM allowed us to determine if CaM could bind MLCK after exposure to ACTs of varying duration. Figure 3.7B shows time course of CaM-MIANS fluorescence as the N-terminal of CaM transiently binds to MLCK when 200 μM Ca²⁺ was rapidly mixed with 200 nM CaM-MIANS + 400 nM MLCK in the presence of 1, 2 or 5 mM EGTA. In order to model the transient binding of CaM-MIANS to MLCK an association rate of the complex was fixed at 6.7x10⁷ M⁻¹ s⁻¹, consistent with previously determined association rates of CaM for smooth and skeletal muscle MLCK of 2.8x10⁷ M⁻¹ s⁻¹ - 4.6x10⁷ M⁻¹ s⁻¹, respectively (74, 104) (see table 3.1). The model also fixed CaM’s N-terminal Ca²⁺ association and dissociation rates of 1.6 x10⁸ M⁻¹ s⁻¹ and 405/s, respectively; and fixed CaM’s N-terminal Ca²⁺ association and dissociation rates when bound to MLCK of 6.7 x10⁶ and 0.031/s, respectively. The modeling assumes that the apo N-terminal of CaM (not bound to Ca²⁺) does not bind MLCK. The modeling also assumes that the rate limiting step of MLCK dissociation is Ca²⁺ dissociation from the N-terminal of CaM in the Ca²⁺-CaM-MLCK complex, consistent with the RS-20 data. The modeling shows that a 0.6 ms hw ACT (Figure 5.2, 2 mM EGTA trace) allowed ~60% of the N-terminal of CaM to transiently complex with MLCK, which then dissociated at a rate of ~1.5/s as EGTA removed the Ca²⁺ from the protein complex. Figure 5.2 also shows the
transient binding of the N-terminal of CaM to MLCK following ACTs with hws of 0.2 and 1.1 ms. A shorter, 0.2 ms hw, ACT (Figure 5.2, 5 mM EGTA trace) resulted in ~40% of the CaM-MIANS transiently binding to MLCK, with similar kinetics as the 2 mM EGTA trace. A longer, 1.1 ms hw, ACT (Figure 5.2, 1 mM EGTA trace) resulted in ~80% of the CaM-MIANS transiently binding to MLCK, again with similar kinetics. As can be seen from the modeling, these simulations approximate the data quite well and confirm the predicted rates of Ca$^{2+}$-CaM association to MLCK.
Figure 5.2: The binding of the N-terminal of CaM to MLCK during artificial Ca\(^{2+}\) transients of different duration.

The simulations (using KSIM) show the time course of the rise and fall of N-terminal CaM binding to MLCK when 100 µM Ca\(^{2+}\) was rapidly mixed with 100 nM CaM + 200 nM MLCK in the presence of 0.5, 1 or 2.5 mM EGTA (after mixing concentrations). At time = 0, the initial [Ca\(^{2+}\)], [EGTA], [CaM] and [MLCK] were set to the concentrations representing the amount of species that would be present in the stopped-flow apparatus just after mixing was complete (assuming infinitely fast mixing time). The simulation with no EGTA present (No EGTA trace) in which CaM and MLCK were mixed with 100 µM Ca\(^{2+}\), showed that nearly 100% of N-terminal of CaM bound Ca\(^{2+}\) and MLCK. Ca\(^{2+}\) on and off-rates from EGTA and CaM in the presence and absence of MLCK were used as listed in table 3.1.
LIST OF REFERENCES


135


137


