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TUNING CALCIUM BINDING AFFINITIES WITH RELATED BIOLOGICAL FUNCTIONS OF CALMODULIN AND DESIGNING PROTEIN BASED CONTRAST AGENT

by

JIE JIANG

Under the Direction of Professor Jenny J. Yang

ABSTRACT

Calmodulin (CaM) is a ubiquitous intracellular protein that regulates biological activities of numerous enzymes and ion channels. Upon responding Ca\(^{2+}\) concentration change, Ca\(^{2+}\)-dependent CaM activates the hydrolyzation of cGMP by PDE and Ca\(^{2+}\) releasing channel activity of ryanodine receptor. In this dissertation, a series of CaM variants were engineered to enhance Ca\(^{2+}\) binding affinities by increasing the number of negative charged residues in individual EF-hand. The capability of shifting the biphasic Ca\(^{2+}\)-activation profile of RyR1 is significantly altered by changing Ca\(^{2+}\) binding affinity of CaM at the C-terminal. This indicates that examining Ca\(^{2+}\)-CaM affinity is a valid strategy to tune the activation profile of CaM-regulated ion channels. To further understand interactions between CaM and RyR1, NMR was used to determine their binding mode. To dissect roles of structural components of CaM in metal binding and regulation of biological functions of target proteins, we created half-CaMs and Del-CaM. Binding
affinities of these variants to Ca$^{2+}$, Tb$^{3+}$ and Gd$^{3+}$ were determined by fluorescence spectroscopy; functional studies were conducted using single channel analysis and PDE function assay.

Another objective of my dissertation is to design a protein based contrast agent for molecular imaging. CaM was selected as the scaffold protein for designing Gd$^{3+}$ based MRI contrast agent by modifying metal binding sites as well as grafting a biomarker peptide into the linker region to specifically target cancers with efficient and optimized modifications. The physical kinetic properties and animal imaging effects of these designed contrast agents were investigated by various methods.

INDEX WORDS: Calmodulin, Calcium, RyR1 channel, Phosphodiesterase, MRI, Contrast agent, Relaxivity, Biomarker
TUNING CALCIUM BINDING AFFINITIES WITH RELATED BIOLOGICAL FUNCTIONS OF CALMODULIN AND DESIGNING PROTEIN BASED CONTRAST AGENT

by

JIE JIANG

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the College of Arts and Sciences Georgia State University 2011
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LIST OF ABBREVIATIONS

CaM  Calmodulin
CD   Circular Dichroism
DTT  1,4-dithio-DL-threitol
EGTA Ethylene glycol tetraacetic acid
ER   Endoplasmic reticulum
FRET Fluorescence resonance energy transfer
FPLC Fast performance liquid chromatography
GST  Glutathione-S-transferase
HEPES 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid
HSQC Heteronuclear single quantum coherence
ICP  Inductively coupled plasma
IHC  Immunohistochemistry
IPTG Isopropyl -D-thiogalactoside
K\textsubscript{d} Dissociation constant
LB   Luria-Bertani medium
MALDI Matrix-assisted laser desorption/ionization
MOPS 3-(N-morpholino)propanesulfonic acid
MRI  Magnetic Resonance Image
MS   Mass spectrometry
NIR  Near Infrared
NMR Nuclear magnetic resonance
PBS Phosphate buffer saline
PCR Polymerase chain reaction
PDE Phosphodiesterase
PEG Polyethylene glycol
ProCA Protein based contrast agent
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
UV   Ultraviolet
1. Introduction

1.1. Biological functions and structure of calmodulin

Calmodulin (CaM) is an acidic (pI ~4.0) intracellular Ca\(^{2+}\) signaling protein comprised of 148 amino acids with a molecular weight of 16.7 kDa. It was first discovered as a cyclic nucleotide phosphodiesterase activator protein (PAF) in the brain and heart\(^1\). It was subsequently rediscovered several times, since many Ca\(^{2+}\)-dependent cellular processes were eventually shown to involve the same Ca\(^{2+}\)-binding protein. The name CaM, was first suggested by W. Y. Cheung. Calcium-free CaM is referred to as apo-calmodulin (ApoCaM). It was then rediscovered as a calmodulin by Saimi Y, et al\(^2\).

Calmodulin is widely distributed in nature. It is a ubiquitous intracellular calcium binding protein located in all mammalian cells including vertebrates and plants \(^3,4\). The sequences of CaM are highly conserved in all eukaryotic proteins (Figure.1.1). All known vertebrate CaMs are identical, and all metazoan CaMs are essentially the same. CaM of paramecium is 94% similar to the mammalian counterpart and that of human, bovine and rat are the same \(^5\). In mammals, there are three genes in each species which are 80% identical in nucleotide sequence but encode 100% identical protein sequence \(^6\). This high degree of conservation may be essential for the maintenance of interaction with a diverse family of CaM-binding proteins \(^7\).
**Figure 1.1.** PDB structures of apo, holo form calmodulin and sequence alignment.

**A.** Structures of ApoCaM (PDB code 1cfc) and Ca$^{2+}$-CaM (PDB code 3cln).

**B.** Sequences alignment of CaM from different sources. Amino acids that are not different from the human sequence are shown in red.
As shown in Figure 1.1, CaM is composed of three structural components: the N- and C-terminal domains with two EF-hand motifs in each domain, and a flexible linker which connects these two domains. EF hand is a helix-loop-helix structural domain found in the large family of Ca\(^{2+}\)-binding proteins. It was first discovered from parvalbumin, a Ca\(^{2+}\) buffering protein containing three EF hand motifs and involved in muscle relaxation via its Ca\(^{2+}\)-binding property. An EF hand motif contains a helix-loop-helix structure, in which the Ca\(^{2+}\) ions are coordinated by ligands within the loop. It consists of two \(\alpha\) helices positioned perpendicular to one another and linked by a short loop region (typically 12 amino acids). This loop will form a binding pocket to bind Ca\(^{2+}\) (Figure 1.2).

In the EF hand binding pocket is coordinated in a pentagonal bipyramidal configuration. The six residues contribute to the binding are located in positions 1, 3, 5, 7, 9 and 12; these residues are denoted by X, Y, Z, \(-Y\), \(-X\) and \(-Z\) as a space axis. Glu or Asp at position 12, named as an anchor, provides two oxygens for binding Ca\(^{2+}\) (see detailed discussion in Chapter 3). Ca\(^{2+}\) is bound with oxygens from both the protein backbone and side chains. Charge-charge interaction is the major driven force for Ca\(^{2+}\) binding to this EF hand pocket. In most proteins, the sixth residue in the loop is found to be glycine due to the conformational requirements of the backbone. The remaining residues are usually hydrophobic and form a hydrophobic core that stabilizes the two \(\alpha\)-helices.
Figure 1.2. EF hand structures of Ca\(^{2+}\) binding sites in CaM.

A). EF-hand motif contains a helix-loop-helix topology, and looks like the spread thumb and forefinger of the human hand topology.

B). Ca\(^{2+}\) binding pocket of the EF hand motif with a pentagonal bipyramidal configuration.

Ca\(^{2+}\) is one of the most important second messengers in eukaryotic cells. Temporal and spatial changes of the Ca\(^{2+}\) concentration in different compartments of cells affect the regulation of cellular signaling. Through signal stimulation or alteration in the membrane potential, the cytosolic Ca\(^{2+}\) concentration rises from 10\(^{-7}\) to 10\(^{-5}\) M. As shown in Figure 1.3, CaM is capable of transducing the intracellular Ca\(^{2+}\) signal changes into a number of diverse cellular events, including apoptosis, cell differentiation and cell proliferation through its reversible or irreversible binding to Ca\(^{2+}\). The flexible conformational changes allows interactions with more than 100 functional target enzymes, cellular receptors and ion channels. Currently, the Yang laboratory is studying several calcium binding proteins that are regulated by CaM, such as ryanodine receptor, gap junction, phosphodiesterase (PDE), and G-protein coupled receptor (GPCR). Section 1.2 introduces the ryanodine receptor and PDE as model systems for our studies in this dissertation.
Among myriads of Ca\textsuperscript{2+}-binding proteins which modulate cell signalling, CaM plays an essential role and is extensively studied due to its ubiquitous expression in eukaryotes in addition to its versatile ability to activate or inhibit more than 300 functional enzymes, cellular receptors and ion channels \textsuperscript{10,11,12}. Although quite a few studies have been done on CaM, there are still large numbers of unknown scopes for this protein. For example, the binding sites of CaM to distinct enzymes are different, which suggests the function of CaM is changed by changing the Ca\textsuperscript{2+} binding affinity in each binding site.

This study aims to investigate the structural and functional characteristics of CaM.

Structural data from the protein data bank (PDB) indicates that CaM adopts different structures in the apo- and holo- forms. More hydrophobic residues are exposed and a flexible linker as a result of binding to Ca\textsuperscript{2+}. Most enzymes that are activated by Ca\textsuperscript{2+}-CaM require that three or four Ca\textsuperscript{2+} molecules be bound. Ca\textsuperscript{2+} induced conformational rearrangements within CaM, resulting from the complex formed by binding of Ca\textsuperscript{2+} may provide a mechanism for transferring intracellular signals to diverse target enzymes. \textbf{Figure 1.3} summarizes the interacting modes of CaM with target enzymes. Upon binding to Ca\textsuperscript{2+}, CaM adopts a more helical structure, which makes it easier to bind the target proteins. By binding to the peptide on the targeting enzymes, the two domains of CaM will either wrap closer, such as skeletal muscle myosin light chain kinase (skMLCK) \textsuperscript{13}, or remain extended, as seen in the Ca\textsuperscript{2+} pump and IQ motif of myosin V \textsuperscript{14,15}. 
Figure 1.3. Interaction modes of CaM to various peptides in binding sites of its target enzymes.

CaM interacts with the target enzymes in two major modes: the collapsed mode (1-7,1-10,1-14,1-16) and the extended mode.
Figure 1.4 shows that the cytosolic Ca$^{2+}$ concentration rises from $10^{-7}$ to $10^{-5}$ M through signal stimulation or alteration in the membrane potential. CaM undergoes a conformational change and interacts with numerous target proteins, such as ryanodine receptor and IP3 receptor at the ER, gap junction proteins at the plasma membrane, and phosphadieasterase in the cytosol to modulate Ca$^{2+}$ signaling. Insights into the role of Ca$^{2+}$ binding to CaM with respect to the regulation of targeting enzymes have been reported using site-direct mutations, for example, replacing the anchor glutamate within the EF-hand loop with other hydrophobic residues. Therefore, it may be possible to selectively enhance a specific CaM function on targeting enzymes by manipulating the affinity of each EF-hand loop in CaM.

The Ca$^{2+}$ concentration range to which CaM responds increases as a result of the 10-fold higher Ca$^{2+}$ affinity of the C-terminal domain (Ca$^{2+}$-binding sites III and IV) compared to the N-terminal domain (Ca$^{2+}$-binding sites I and II). These different Ca$^{2+}$ affinities contribute to CaM’s functional bifurcation which allows the two domains of CaM to serve different functions. For example, Ca$^{2+}$-dependent facilitation of Ca$_{v}$2.1 voltage-gated Ca$^{2+}$ channels requires Ca$^{2+}$ binding to CaM’s C-terminal while Ca$^{2+}$-dependent inhibition of these channels requires Ca$^{2+}$ binding to CaM’s N-terminal. Further, the two lobes of CaM may separately decode global and local Ca$^{2+}$ signals. Therefore, one strategy to manipulate cellular responses to Ca$^{2+}$ signals may be to modify the Ca$^{2+}$ affinity of the protein which decodes the signal. Because of its functional bifurcation, CaM is an attractive target. By manipulating the affinity of one domain of CaM it may be possible to selectively enhance a specific CaM function.
Figure 1.4. Cellular distribution and targeting enzymes of calmodulin (CaM).

Calmodulin is distributed across different cellular compartments including nuclei, ER and mitochondria. In addition, CaM has been reported to interact with membrane proteins such as ion channels, gap junctions and pumps as well as cytoskeletal proteins, and quite a few enzymes in the cytoplasm.
1.2. Ryanodine receptor and PDE are regulated by CaM

Ryanodine receptor Ca\^{2+} release channels from the efflux pathway for the mobilization of Ca\^{2+} stores from the sarco/endoplasmic reticulum (Figure 1.4)\textsuperscript{32}. Three types of ryanodine receptors have been described so far (Figure 1.5). The skeletal muscle isoform of the ryanodine receptor (RyR1) is essential for Ca\^{2+} release from the sarcoplasmic reticulum (SR) to activate contraction\textsuperscript{33}. RyR1 is a homotetramer composed of four 565 kDa subunits, each with a single high-affinity CaM binding site\textsuperscript{34,35,36}. Cardiac muscle cell Ca\^{2+} release channel ryanodine receptor 2 (RyR2) is a homotetramer consisting of a COOH-terminal transmembrane assembly and a massive cytoplasmic NH\textsubscript{2}-terminal domain with a molecular weight ~ 2000 kDa. The physiological importance of regulation of RyR2 by CaM is currently understood. More recently, the third gene RyR3 has been identified in rabbit brain\textsuperscript{37}, mink lung epithelial cells \textsuperscript{38}, and human Jurkat T-cell\textsuperscript{39}. Based on the fact that RyR3 has been detected in specific region of the brain, e.g. hippocampus, thalamus, and corpus striatum, it may have an important role in specific brain functions. Presently, however, the properties of RyR3 are still unclear.
Figure 1.5. Comparison of 3D reconstructions of RyR1, RyR2, RyR3.

(A) The solid body representations of RyR3 (blue), RyR1 (green), and RyR2 (red) viewed onto the cytoplasmic surface. Note conservation of domain architecture. (B) RyR3 superimposed with the major differences (yellow) obtained when the 3D reconstruction of RyR3 is subtracted from that of RyR1. Arrows indicate the main difference which is tentatively attributed to the D2 region which is absent from RyR3 (Wagenknecht T, Samsó M)\textsuperscript{40}. 
In vitro, the RyR1 channel opening exhibits a biphasic Ca\textsuperscript{2+} dependence attributed to high- and low-affinity Ca\textsuperscript{2+} binding sites which when occupied will, respectively, activate or inhibit channel opening \textsuperscript{41}. At micromolar Ca\textsuperscript{2+} concentrations, CaM inhibits both RyR1 and RyR2. However, at sub-micromolar Ca\textsuperscript{2+} concentrations, CaM activates RyR1 (Figure 1.6A) but inhibits RyR2 \textsuperscript{42} (Figure 1.6B). CaM shifts the Ca\textsuperscript{2+} dependence of the RyR1 opening to lower Ca\textsuperscript{2+} concentrations, effectively increasing channel opening at sub-micromolar Ca\textsuperscript{2+} level and inhibiting the channel in micromolar Ca\textsuperscript{2+} concentration\textsuperscript{43}. The switch from a channel activator to a channel inhibitor is due to Ca\textsuperscript{2+} binding to CaM as Ca\textsuperscript{2+} insensitive mutants of CaM activate but do not inhibit RyR1\textsuperscript{36}. The Ca\textsuperscript{2+}-induced conversion of CaM from a RyR1 activator to a channel inhibitor suggests that the RyR1 activation profile might be manipulated by tuning the CaM Ca\textsuperscript{2+} affinity. This strategy requires that the CaM Ca\textsuperscript{2+} binding site(s) acting as the RyR1 regulatory switch be identified and the Ca\textsuperscript{2+} affinity of the specific site be modified without significantly altering the RyR1-CaM functional interaction. We chose RyRs as a model system to study the binding properties of CaM to its target enzyme because of extensive studies of the structure, function, importance of RyR in regulation of Ca\textsuperscript{2+} signaling and homeostasis and mutations in RyR related to many human diseases such as malignant hyperthermia (MH) and Duchene muscular dystrophy (DMD).
Figure 1.6. Regulation of CaM on the RyR1 and RyR2 channels in a Ca\(^{2+}\) dependent way. CaM lowered both RyR1 (green line in A) and RyR2 (red line in B) channels open probability from low micromolar to submicromolar Ca\(^{2+}\) concentrations by decreasing the number of channel events and increasing the duration of close times.

A). CaM activate the RyR1 channel opening at low Ca\(^{2+}\) concentration and inhibit the channel at higher Ca\(^{2+}\) concentration.

B). RyR2 channel is mainly inhibited by CaM with low activity in the presence of Ca\(^{2+}\).
Cyclic nucleotide hydrolysis activity was first characterized by Sutherland and Rall following their discovery of cyclic adenosine 3′:5′ – monophosphate (cAMP)\textsuperscript{44}. In general, the cAMP and cyclic guanosine 3′:5′ – monophosphate (cGMP) are intracellular secondary messengers that exhibit rapid concentration changes in response to a wide variety of cell – specific stimuli. The concentrations of both cAMP and cGMP are determined by the relative synthetic activity of adenylate cyclase and the degradative activity of cyclic nucleotide phosphodiesterase (PDE). Hydrolysis of the cyclic nucleotides by PDE is a unique mechanism for their degradation. The substrate specificities and affinities, and the sensitivities of PDEs isolated from different tissues are distinguished. Three major forms of PDE were classified into three groups based on their functions: cAMP PDE, cGMP PDE and those with dual substrates for both cAMP and cGMP\textsuperscript{45}. The activity of PDE is Ca\textsuperscript{2+}/CaM dependent. Since the role of Ca\textsuperscript{2+}/CaM in PDE activity has been studied in several research groups. We chose PDE1 as a model system to test the roles of structural components of CaM in activation of PDE (Chapter 6).
Figure 1.7. Functional regulation of phosphodiesterase in the cytosol.
Phosphodiesterase (PDE) catalyzes the hydrolysis of cGMP/cAMP to the corresponding 5'-nucleoside monophosphates, thereby terminating cyclic nucleotide signaling. The buildup of cGMP stimulates the uptake of Ca$^{2+}$ into the SR/ER, reducing cytoplasmic Ca$^{2+}$, promoting relaxation of vascular smooth muscle and Vasodilation$^{46}$. 
1.3. Designing protein based MRI contrast agent

Magnetic resonance imaging (MRI) is a relatively new technology first developed by Lauterbur, P.C et.al in the University of Aberdeen, UK. The first MR image was published in 1973 using MnSO₄ as the magnetic agent⁴⁷,⁴⁸ and the first cross-sectional image of a living mouse was acquired by Lai et.al through a 2D image⁴⁹. The first studies performed on humans were published in 1977 with video images generated of a cross-section through the chest at the level of the eighth thoracic vertebra⁵⁰,⁵¹. Magnetic resonance imaging (MRI) has been applied as an advanced imaging technique in both clinical and research studies. MRI has emerged as one of the most important tools for diagnosing various disease including cancers due to several advantages, such as the high resolution, the ability to map three-dimensional structures and the function of soft tissues in vivo without any limitation of depth. Clinical MRI takes advantages of the magnetic properties of protons (mostly from water) and their interaction with both the strong external magnetic field and the electro-radio wave to produce highly detailed images of the human body. In addition, the application of contrast agents particularly enhances the MRI signals among different tissues and organs. Contrast agents can be defined either based on their physical properties or according to their applications. For example, T₁ / T₂ weighted contrast agents, or non specific / specifically molecular-targeted contrast agents. MR imaging can be helpful for characterizing normal tissue and diseased ones by giving different contrast in the images. In particular, gadolinium-enhanced MR imaging can help distinguish between benign and malignant tumors. However, most of the clinically contrast agents use Gd³⁺ with small organic chelators which often include drawbacks such as low relaxivities, high toxicity and short retention.
time in the human body. As a result, there is a strong need to develop novel classes of contrast agents with high sensitivity, high relaxivity and low toxicity.

Rapid development in biomarkers and molecular imaging allows monitoring disease progression at a molecular level. Several targeted therapies against tumor biomarkers such as HER2 and gastric releasing peptide (GRP) receptor largely expressed at tumor cell surface have been developed. To facilitate the application of MRI to molecular imaging of cancer progression and evaluate drug effect, it is important to develop targeted contrast contrasts. To satisfy the essential requirement for targeting biomarkers expressed on tumor cell surface, we need to significantly improve molecular relaxivity of the contrast agents in addition to enhancing the MRI signal for targeted disease biomarkers.

Based on the criteria of designing contrast agent (for details please see Chapters 7-9) and our detailed investigation of the properties of metal binding and selectivity of CaM and key determinants for molecular recognition of calmodulin and its target proteins, we select CaM as a strong candidate for the development protein based contrast agents by protein engineering. Our project initially entailed enhancing the metal bind affinity of CaM to Gd$^{3+}$ and selectivity over physiological metal ions by protein engineering. Next, we reduced calmodulin’s endogenous recognitions to its target proteins by site directed mutagenesis and PEGylation. Further, we carefully investigated their relaxation properties as protein based contrast agents. Finally, we further developed targeted contrast agents for the prostate and breast cancers using gastrin releasing peptide and HER-2 affibody as a targeting moiety against their corresponding biomarkers respectively.
1.4. The objective of this dissertation

The first objective of this research is to study the Ca\(^{2+}\) binding affinity to one of the most significant intracellular proteins, CaM, and the effects on the function of RyR. A series of CaM variants designed to enhance the affinity of specific Ca\(^{2+}\) binding sites was engineered by increasing the number of negative charge from acidic residues within individual EF-hands, which we propose will thereby affect the Ca\(^{2+}\)-activation profile of RyR1. The functional effects of these mutants were assessed via their effects on the Ca\(^{2+}\)-activation profile of RyR1. The experimental results indicate that tuning CaM's Ca\(^{2+}\)-binding affinities offers a potential strategy for tailoring the activation profile of CaM targets.

The second objective of this dissertation is to understand the roles of the structural components of the two domains and the linker region of CaM. We think they may play diverse roles in both metal binding and biological functions of CaM considering that CaM displays a tremendous conformational change through binding to Ca\(^{2+}\) and the fact that N- and C- terminal domains of CaM have distinct binding affinities to Ca\(^{2+}\). More interestingly, previous studies have reported that lanthanides may occupy Ca\(^{2+}\) binding sites in a reverse order to Ca\(^{2+}\) for the N- and C- domains of CaM\(^{52}\). To probe binding affinities, CaM mutants were created including half-CaM constructs (either the Isolated N- or C- terminal of CaM), and a deletion variant where five residues in the central linker were removed (Del-CaM) by molecular cloning. The binding affinity between the CaM mutants and several metals, including Ca\(^{2+}\) and Tb\(^{3+}\) / Gd\(^{3+}\) were determined mainly by fluorescence spectroscopy; Functional studies were conducted using single channel analysis and PDE function assay.
The third objective of my dissertation was to design a protein based contrast agent for molecular imaging. CaM was selected as the scaffold protein for designing Gadolinium based MRI contrast agent by modifying metal binding sites as well as grafting a biomarker peptide into the linker region to specifically target cancers with efficient and optimized modifications. The physical kinetic properties and animal imaging effects of these designed contrast agents were investigated by a variety of techniques.

1.5. The overview of this dissertation

First, we designed a series of mutations from CaM based on the hypothesis that the Ca$^{2+}$ binding affinity to CaM can be tuned by optimizing the number of charged residues. Furthermore, the significant changes in biological functions when CaM and its mutants interact with RyR1 suggest that tuning Ca$^{2+}$ binding affinity will affect the related functions. Second, isolated CaMs were created to further understand the functional role each domain in CaM plays as well as the hinge linker. Both structural and functional properties were determined comparably. Third, the structures of CaM and peptide complex in both apo- and Ca$^{2+}$ conditions were analyzed through NMR experiments. Finally, protein based contrast agents (ProCAs) with multiple metal binding sites were designed. These ProCAs indicated high relaxivities and high Gd$^{3+}$ binding affinities. The enhancement in MR imaging also proves that they are potential contrast agents with high specificity targeting properties to various cancers.
Chapter 3 discusses the single mutations in each EF-hand loop of CaM designed to tune the activity of Ryanodine receptor. Ca\(^{2+}\) binding affinities of these mutants were determined by domain specific fluorescence titration.

Chapter 4 focuses on analyzing the binding mode of CaM with ryanodine receptor using a peptide model. NMR is the essential method in this discussion.

In Chapter 5 and Chapter 6, we use ryanodine and phosphodiesterase as examples to dissect the structural components of CaM to the regulation of biological functions.

In Chapter 7 and Chapter 8, we try to apply CaM and its structural components as a protein based MRI contrast agent.

In Chapter 9, we optimized the protein based MRI contrast agent using PEGylation to increase the stability and relaxivity as well as enhance the MR images.
Table 1.1 Summarized table of CaM variants studies in this dissertation.

<table>
<thead>
<tr>
<th>CaM variants</th>
<th>Change in CaM</th>
<th>Purpose</th>
<th>chapter</th>
</tr>
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<td>wt CaM</td>
<td></td>
<td>Effect on the Ca(^{2+}) binding affinity in the N- terminal</td>
<td>3</td>
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<tr>
<td>T26D</td>
<td>Increase one negative charge in loop I</td>
<td>Effect on the Ca(^{2+}) binding affinity in the N- terminal</td>
<td>3</td>
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<tr>
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<td>Increase one negative charge in loop II</td>
<td>Effect on the Ca(^{2+}) binding affinity in the N- terminal</td>
<td>3</td>
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<tr>
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<td>Effect on the Ca(^{2+}) binding affinity in the C- terminal</td>
<td>3</td>
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<td>Q135D</td>
<td>Increase one negative charge in loop IV</td>
<td>Effect on the Ca(^{2+}) binding affinity in the C- terminal</td>
<td>3</td>
</tr>
<tr>
<td>N60D+N97D</td>
<td>Increase one negative charge in loop II and III</td>
<td>Design protein based contrast agent</td>
<td>7</td>
</tr>
<tr>
<td>T27W</td>
<td>Introduce Trp in N-terminal</td>
<td>Monitor Trp fluorescence signal</td>
<td>N/A</td>
</tr>
<tr>
<td>Y99W</td>
<td>Introduce Trp in C-terminal</td>
<td>Monitor Trp fluorescence signal</td>
<td>N/A</td>
</tr>
<tr>
<td>D20A</td>
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<td>Effect on the Ca(^{2+}) binding affinity in the N- terminal</td>
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<tr>
<td>D56A</td>
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<td>Effect on the Ca(^{2+}) binding affinity in the N- terminal</td>
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<td>Effect on the Ca(^{2+}) binding affinity in the C- terminal</td>
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<td>D129A</td>
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<td>Effect on the Ca(^{2+}) binding affinity in the C- terminal</td>
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<td>N-CaM</td>
<td>Isolated N-terminal domain of CaM</td>
<td>Dissect the functional contributions of CaM structural components; Design protein based contrast agent</td>
<td>5,6,8</td>
</tr>
<tr>
<td>C-CaM</td>
<td>Isolated C-terminal domain of CaM</td>
<td>Dissect the functional contributions of CaM structural components; Design protein based contrast agent</td>
<td>5,6,8</td>
</tr>
<tr>
<td>Del-CaM</td>
<td>Delete five residues in the helical linker region</td>
<td>Dissect the functional contributions of CaM structural components; Design protein based contrast agent</td>
<td>5,6,8</td>
</tr>
<tr>
<td>N60D-N97D- affibody-CaM(ProCA22)</td>
<td>Insert affibody between AA 80 and 81 of CaM</td>
<td>Design protein based contrast agent with HER-2 target peptide</td>
<td>7</td>
</tr>
</tbody>
</table>
2. Materials and methods

2.1. Molecular cloning and protein engineering

2.1.1. Site mutation of CaM in single EF-hand loop

Mutations targeting specific CaM Ca$^{2+}$-binding single site in each EF-hand loop were introduced using a polymerase chain reaction (PCR) and further purified through the QuikChange mutagenesis kits (Stratagene, La Jolla CA). These mutations were verified by DNA sequencing respectively.

2.1.2. RyR1 mini domain peptide containing CaM binding site

The mini domain peptide of RyR1 with the two reported binding sites for CaM (RyR1 1975-1999 and RyR1 3614-3643) was inserted into pGEX-2T vector after the BamHI restriction site with GGSGG linker between these two peptides. New constructed DNA was purified by using a standard PCR method and sequencing by GENEWiz, INC.

2.2. Protein over-expression and purification

2.2.1. Expression and purification of CaM and its variants

Recombinant rat CaM and its mutants were over expressed in *E.coli* strain BL21 (DE3) pLysS using the pET-7 vector $^{53,54}$ expressed in the Luria Bertani (LB) medium with 100 mg/L ampicillin for 4 hrs at 37 °C after induced with IPTG. Proteins were purified by phenyl-sepharose chromatography$^{55}$. Protein concentrations were determined based on tyrosine absorbance at 277 nm and using the molar extinction coefficient 3030 M$^{-1}$ cm$^{-1}$ with Beer–Lambert equation$^{56}$. 

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2.2.2. Expression and purification of RyR1 mini domain

The mini domain peptide of RyR1 in the CaM binding sites was constructed in the plasmid pGEX-2T and expressed in *E. coli* cell strain BL21 (DE3) in LB medium with 100 mg/L ampicillin and was induced by IPTG when the OD reached to ~ 0.6. The purification procedures followed the protocols for GST-fusion protein purification using glutathione sepharose 4B beads (GE Healthcare). The GST-tag fused to proteins was cleaved on beads by thrombin. The purified peptide was confirmed by MALDI-TOF-MS in the Advanced Biotechnology Core Facilities of Georgia State University. The concentrations of engineered mini domain variants were determined using $\varepsilon_{280}$ of 8250 M$^{-1}$cm$^{-1}$.

2.2.3. Expression and purification of $^{15}$N isotopic labeled proteins

$^{15}$N isotopically labeled proteins were expressed in a medium consisting of K$_2$HPO$_4$, KH$_2$PO$_4$, MgSO$_4$ and (NH$_4$)$_2$FeSO$_4$. $^{15}$NH$_4$Cl was added into the mini (200 ml) overnight and full (1 L) expressed medium as a source for the isotopic nitrogen labeling protein. Temperature was reduced from 37 to 25 degree (or even lower, such as 16 degree) after induction by IPTG when the optical density ($OD_{600}$) reached 0.6 and then expressed overnight. The collected cell pellets were purified using the same method as normal proteins.
2.3. Circular Dichroism Spectroscopy

Circular dichroism (CD) spectra were obtained in the far UV (190 - 260 nm) wavelength region on a Jasco-810 spectropolarimeter at room temperature using a Tris-KCl buffer (10 mM Tris pH 7.4, 100 mM KCl) in a 1 cm light-path length quartz cuvette. All spectra are evaluated with the background subtracted and were comprised of an average of at least 10 scans. Protein concentrations are used for 10 µM. The spectra with Ca\(^{2+}\) and without Ca\(^{2+}\) were collected respectively to compare the conformational changes due to the binding with Ca\(^{2+}\). To determine the structural changes of RyR\(_{3614-3643}\) upon binding to CaM. Far UV CD spectra of RyR1 peptide alone were subtracted from the spectra of CaM plus the peptide.

2.4. Equilibrium Calcium Titrations

The CaM lobe-specific Ca\(^{2+}\)-affinities were determined by monitoring the intrinsic fluorescence of phenylalanine (\(\lambda_{ex} = 235\) nm, \(\lambda_{em} = 280\) nm) and tyrosine (\(\lambda_{ex} = 277\) nm, \(\lambda_{em} = 320\) nm) which are sensitive indicators of Ca\(^{2+}\) binding to CaM's N- and C-terminal lobes, respectively \(^{57,58}\). The lobe specific Ca\(^{2+}\)-affinities of CaM bound to the putative RyR1 CaM binding domain, RyR1\(_{3614-3643}\) \(^{35}\), were similarly determined but with tyrosine excited at 265 nm and emission monitored at 292 nm to avoid interference from the peptide intrinsic tryptophan fluorescence. All spectra were collected at ambient temperature using a PTI spectra fluorometer with a 1 cm path length quartz cuvette. Ca\(^{2+}\) stock solution (15 or 50 mM) was titrated into 5 or 10 µM CaM in a buffer containing 50 mM HEPES, 100 mM KCl, 5 mM NTA, 0.05 mM EGTA, pH 7.4. Free Ca\(^{2+}\)
concentration was determined by using Oregon Green 488 BAPTA – 5N (0.2 µM) according to Equation 1.

Fluorescence signal decrease of CaM by monitoring the phenylalanines located in the N-terminal domain.

\[
[Ca^{2+}]_{\text{free}} = K_d \frac{F - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} \quad \text{Equation 1}
\]

Where \( F \) is the fluorescence at each titration point, \( F_{\text{min}} \) and \( F_{\text{max}} \) are the fluorescence intensities at zero \( Ca^{2+} \) and saturating \( Ca^{2+} \) respectively. The Oregon Green dissociation constant for \( Ca^{2+} \) is 21.7 µM (\( \lambda_{\text{ex}} = 495 \text{ nm}, \lambda_{\text{em}} = 520 \text{ nm} \)) [30]. Titration data were fit with a nonlinear Hill equation (Equation 2).

\[
f = \frac{[Ca^{2+}]^n}{K_d^n + [Ca^{2+}]^n} \quad \text{Equation 2}
\]

Where \( f \) is the relative fluorescence change, \( K_d \) is the dissociation constant of \( Ca^{2+} \) to CaM, \( n \) is the Hill coefficient, and \([Ca^{2+}]\) is the free \( Ca^{2+} \) concentration.
2.5. Nuclear Magnetic Resonance Spectroscopy

2.5.1. $^1$H NMR spectra

One-dimensional $^1$H NMR spectra were recorded on a Varian 500 MHz NMR spectrometer with a spectral width of 6600 Hz. Samples with concentration of 100 μM were prepared in 10 mM Tris-100 mM KCl, 10% D$_2$O at pH 6.8. Ca$^{2+}$ from a stock solution was gradually added into the NMR sample tube and the temperature was maintained at 37 °C. The program FELIX98 (MSI) was used to process NMR data with an exponential line broadening of 2 Hz window function and the suppression of water signal with a Gaussian deconvolution function with a width of 20.

2.5.2. CaM – RyR1 binding mode determination by HSQC NMR

Heteronuclear Single Quantum Coherence (HSQC) experiment was applied on Varian Inova 600 MHz spectrometer. Apo-CaM was prepared in 100 mM KCl, 5 mM MES, 100 μM NaN$_3$, pH = 6.3, with 10 mM EGTA at 23°C with a final concentration of 0.25 mM. Ca$^{2+}$-CaM was prepared in 10 mM Bis-Tris, 5 mM MES, 100 μM NaN$_3$ buffer, pH = 6.5, with 10 mM CaCl$_2$ at 37 °C, and final concentration was 0.45 mM. All these NMR samples were prepared containing 10% D$_2$O. NMR spectra were acquired with a spectral width of approximately 13 ppm in the 1H dimension and 36 ppm in the $^{15}$N dimension. Two different RyR1 peptides (3614-3643 and 1975-1999) were prepared in mM concentration stock used to titrate into the proteins. The collected NMR data from each titration point were processed by NMRpipe and analyzed using Sparky.
2.5.3. Size determination of CaM and its variants by NMR

The pulse-field-gradient diffusion NMR spectra were collected with a modified PG-SLED pulse sequence on a 600 MHz Varian Inova spectrometer. Each FID was processed with 8k complex data points over a spectral width of ~13 ppm. The pulse field gradient level (G) was arrayed from ~0.2 to ~31.0 Gauss/cm with a pulse gradient time (δ) of 5 ms and a diffusion time (Δ) of 112.5 ms. The data were processed using FELIX (Accelrys).

The relationship between the NMR signal intensity (A) and the diffusion constant (D) follows Equation 3.

\[
A = A_0 \exp \left[ -\left(\gamma \delta G\right)^2 (\Delta - \delta/3)D \right] \quad \text{Equation 3}
\]

Where \(A_0\) is the signal intensity when pulse gradient is not used and \(\gamma\) is the gyromagnetic ratio of the proton. In the data processing, the signal intensity as a function of pulse field gradient level was fitted with Equation 4 using KaleidaGraph 3.5 (Synergy). The values discussed in this article are all from the fitting with linear correlation coefficient ≥ 0.999.

\[
A = A_0 \exp \left(-CG^3\right) \quad \text{Equation 4}
\]

The associate diffusion constants and the hydrodynamic radii were obtained by comparing the C values of different molecules measured under identical conditions using Equation 5 and 6.
\[ \frac{D}{D_0} = \frac{C}{C_0} \quad \text{Equations 5} \]
\[ \frac{R}{R_0} = \frac{C_0}{C} \quad \text{Equations 6} \]

Where \( D_0, R_0 \) and \( C_0 \) are the diffusion constant, hydrodynamic radius, and the measured \( C \) value of lysozyme respectively. The hydrodynamic radius and diffusion constant of lysozyme has been reported previously \(^{60}\).

The gradients were calibrated at 25 °C on the residual \(^1\)H signal in a sample of 99.9% \( \text{D}_2\text{O} \), using the published value of \( 1.902 \pm 0.002 \times 10^{-9} \text{ m}^2 \text{ s}^{-1} \) for the self-diffusion coefficient of HDO at 25 °C \(^{61,62}\).

NMR samples contained 0.23 mM proteins in 10% \( \text{D}_2\text{O} \), 100 mM KCl, and 10 mM imidazole at pH 6.5, with 10 mM \( \text{Ca}^{2+} \) for \( \text{Ca}^{2+}\)-bound or 1 mM EGTA for \( \text{Ca}^{2+}\)-free sample. The intensities of the protein signals were integrated from the methylene and methyl region of ~2 ppm spectral width (-0.2 – 1.8 ppm). The integrate regions for each species were carefully selected to avoid or reduce interferences from buffer signals. The integrated intensities were further normalized to minimize the experimental errors from phase adjustment and baseline correction during processing.

2.6. Single channel recording

SR \( \text{Ca}^{2+} \) release channel protein was purified from SR membrane vesicles as described by Lai et al \(^{34}\). Briefly, SR membranes (1 mg/ml were solubilized in 1 M NaCl, 25 mM piparazine-\( N,N'\)-bis(2-ethanesulfonic acid) (PIPES, pH 7.1), 1.0%
3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% phosphatidylcholine (PC), 2 mM dithiothreitol, 0.8 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.6 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 μg/ml aprotinin for 2 h at 4°C with gentle agitation. Insoluble material was removed by centrifugation at 100,000 g for 30 min. The supernatant was applied to a 5-20% linear sucrose gradients containing 1 M NaCl, 25 mM PIPES (pH 7.1), 0.75% CHAPS, 0.25% PC plus protease inhibitors and centrifuged at 24,500 revolutions per minute (rpm) in a Beckman SW28 rotor at 4°C for 16 h. Gradients were fractionated and tubes containing the RyR were identified by [3H]ryanodine binding. Fractions containing the RyR were pooled, concentrated with an Amicon centirprep 30, aliquotted, frozen in liquid N2 and stored at -80°C until use.

RyR1 mediated currents were recorded as previously described. Muller-Rudin planar lipid bilayers were formed by painting a lipid mixture (phosphatidylethanolamine, phosphatidylserine, and PC in a 5:3:2 ratio by wild type, 50 mg/ml dissolved in n-decane) across a 150-250 μm aperture in a Delrin cup. Recording solution consisted of symmetrical 200 mM KCl, 20 mM MOPS, pH 7.0, 1.0 mM EGTA and 0.8087 mM CaCl2. The cis chamber, to which solubilized channels were added, was connected to the head stage input of an Axoclamp 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). The trans chamber was held at virtual ground. Channels incorporate into the bilayer in a fixed orientation, with the cytoplasmic face toward the chamber into the channels were added, thus the cis chamber corresponds to the cytoplasmic side of the channel. To investigate the effects of CaM and the variants on RyR1 channel activity, aliquots of concentrated wild type or mutant CaM were added to the cis chamber. Single channel currents were recorded at +70 mV (Clampex program, pCLAMP 9.2 software, Axon Instruments). Currents were filtered at 1 kHz and recorded at 5 kHz. Only those channels
that had a conductance of at least 600 pS were used for analysis. Single channel $P_o$ was calculated (Clampfit program, pCLAMP 9.2 software, Axon Instruments) from at least 2 minutes of recording using a half-amplitude threshold. When two channels were present in the bilayer, indicated by current amplitudes of twice the expected magnitude, $P_o$ was estimated as the average $P_o$ of the two channels, calculated as

$$\frac{[P_{o,\text{level}1} + (P_{o,\text{level}2} \times 2)]}{2}. \quad \textbf{Equations 7}$$

Bilayers in which three channels were incorporated were dealt with similarly; recordings were not made from bilayers containing more than three channels.

2.7. Phosphodiesterase (PDE) assay

The Phosphodiesterase 3', 5' – cyclic nucleotide activator – deficient from bovine heart and the substrate 2'-methylanthraniloyl-cGMP (Mant-c-GMP) were purchased from Sigma-Aldrich. All samples were prepared in 10 mM Mops, 100 mM KCl, 200 µM EGTA, 10 mM CaCl$_2$, pH 7.2. The time based fluorescence signal was monitored at excitation wavelength at 282 nm and emission wavelength at 442 nm on the PTI at room temperature. Total scan time was defined as 200 ~ 300 s. The substrate itself was firstly scanned for 50 s; then protein samples of diverse concentrations mixed with the substrate were scanned for another 50 s, followed by addition of PDE with scanning over the last 100 or 200 s. The EC$_{50}$ values were obtained by fitting the catalytic rate of each reaction calculated from the fluorescence intensity.
2.8. **Dye competition method to determine the Gd\(^{3+}\) binding affinity with CaM and its variants**

Gd\(^{3+}\)-binding affinity of wild type and the mutant CaMs were determined by a competition fluorescence titration method with a metal ion indicator Fura-6F (Invitrogen Molecular Probes) applied as a Gd\(^{3+}\) indicator. The fluorescence spectra of Furo-6F were acquired using a fluorescence spectrophotometer (Photon Technology International, Inc.) with a 10 mm path length quartz cell at 25 °C. Fura-6F excitation spectra were acquired at two wavelengths 340 and 380 nm with an emission wavelength at 510 nm. Gd\(^{3+}\)-binding affinity of Fura-6F, \(K_{d1}\), was first determined by a Gd\(^{3+}\) titration with Gd\(^{3+}\) buffer system containing 1 mM nitrilotriacetic acid (NTA). Free Gd\(^{3+}\) concentration was calculated with a NTA Gd\(^{3+}\)-binding affinity of \(2.6 \times 10^{-12}\) M. Fura-6F was mixed with Gd\(^{3+}\) in 1 to 1 ratio for a competition titration accompanied with gradual addition of proper amount of protein aliquots. An apparent dissociation constant, \(K_{\text{app}}\), was estimated by fitting the fluorescence excitation intensity ratio of Fura-6F at 340 and 380 nm with a various concentrations of wt CaM and designed protein based contrast agents by a 1:1 binding model. Gd\(^{3+}\)-binding affinity of CA1.CD2, \(K_{d2}\), was calculated with the following equation:

\[
K_{d2} = K_{\text{app}} \times \frac{K_{d1}}{K_{d1} + [\text{Fura}-6F]} \\
\text{Equation 8}
\]
2.9. Modification of designed protein based contrast agents (ProCA22) by PEGylation

To optimize the protein solubility, blood circulation time, and reduction of immunogenicity, (Methyl-PEO12)$_3$-PEO$_4$-NHS Ester ($P_{40}$), with tri-branches of 12 units PEG was used to react with CaM in the PBS buffer with pH 7.2 at room temperature for one hour. The protein concentration was 100 µM and $P_{40}$ was five times higher than proteins. Samples were run through a 5 ml Mono-Q column on the Amersham Bioscience AKTA-Fast Protein Liquid Chromatography (AKTA-FPLC) instrument to separate the PEGylated protein and free PEG. Solution samples were taken out from the corresponded fractions to run SDS gel and iodine gel respectively.

2.10. Relaxivity determination of protein base contrast agents

Relaxation times ($T_1$ and $T_2$) of vary protein based contrast agents (ProCA), were determined at 1.41 T using a 60 mq Bruker Mini Spec relaxometer. The lattice relaxation time $T_1$ of ProCAs was determined on 1.41 T by inversion recovery pulse sequence and the data were fitted by the equation

$$S = A \times (1 - 2 \times e \left( -T_i/T_1 \right)). \quad \text{Equation 9}$$

The transverse relaxation time $T_2$ of ProCAs was determined through $t_2$-cp_mb pulse sequence which is the Carr-Purcell-Meiboom-Gill (CPMG) spin echo method and the data were fitted by the equation

$$S = A \times e \left( -T_i/T_2 \right). \quad \text{Equation 10}$$
The protein based contrast agent samples (200 μL) with different concentrations were placed in matched glass tubes. The tubes were kept in a water bath at 37°C for 10 min before measurement were taken. Relaxivity $r_1$ and $r_2$ were achieved by liner fitting the relaxation times with corresponding concentrations. The second way to measure relaxivities is to prepare samples of one specific concentration mixed with Gd$^{3+}$ as a proper binding ratio, and then measuring relaxation times $T_1$ or $T_2$ at 37°C. For this method, $r_1$ and $r_2$ were calculated based on

\[
r_1 = \frac{1}{T_1} - \frac{1}{T_{1c}} / C \quad \text{Equation 11}
\]

\[
r_2 = \frac{1}{T_2} - \frac{1}{T_{2c}} / C \quad \text{Equation 12}
\]

Where $T_1$ and $T_2$ are relaxation times with contrast agent and $T_{1c}$ and $T_{2c}$ are relaxation times without contrast agent. C is the concentration of contrast agent in the unit of mM.

To further evaluate the effect of environment on the relaxivities of protein based contrast agent using CaM as a candidate (ProCA22), relaxivities measured and compared for ProCA22 in diverse buffers (10 mM HEPES / 10 mM Tris, pH 7.4) and under the condition of high salt (100 mM KCl or NaCl) or low salt without adding any KCl or NaCl were compared. Moreover, the relaxivities of ProCA22 mimic the in vivo environment mixed with human serum for a number of time points were tested using the same method on 1.41 T relaxometer. In addition, relaxivities of different series of ProCAs were obtained on distinct magnetic field, such as 4.7 T and 7 T. Other mutants of CaM, for example the isolated N- and C- terminal, and linker deleted CaM were also prepared in the same way to measure their relaxivities on 1.41 T.
2.11. Water number measurement by Tb$^{3+}$ luminescence decay

The designed protein based contrast agent samples prepared in H$_2$O buffer system solution were replaced with D$_2$O by first lyophilizing the samples and then redissolving them in D$_2$O. This process was repeated for at least three times. Tb$^{3+}$-luminescent decay in both H$_2$O and D$_2$O were measured to determine the number of water ligands coordinated to Gd$^{3+}$-ProCA22 and ProCA22-P$_{40}$ complex. The Tb$^{3+}$ excited-state lifetime was measured using a fluorescence spectrophotometer (Photon Technology International, Inc.) with a 10 mm path length quartz cuvet at room temperature (22 °C). Following excitation at 265 nm with a XenoFlash lamp (Photon Technology International, Inc.), in a time based decay fluorescence experiment, Tb$^{3+}$ emission was monitored at 545 nm in both H$_2$O and D$_2$O systems. Luminescence decay lifetime was obtained by fitting the acquired data with a mono exponential decay function. The water number coordinated to the Tb$^{3+}$-ProCA22 and ProCA22-P$_{40}$ complexes were then obtained by fitting the acquired $\Delta k_{\text{obs}}$ value to the standard curve.

2.12. Cell targeting of protein based contrast agents

Two human cancer cell lines, SKOV-3 and MDA-MB-231 were selected to examine whether the designed ProCA22 can target to cancer cells. SKOV-3 is an ovarian cancer cell line with estimated $3 \times 10^6$ HER-2 / cell. MDA-MB-231 is a breast cancer cell line with modest HER2 levels ($\sim 3 \times 10^4$ HER-2 / cells). Binding of the Gd-ProCA22 and ProCA22 – P40 to the selected cells was first analyzed by immuno-fluorescence staining using the polyclonal antibody against PEGylated parental protein ProCA22. The cell binding analyses showed that the SKOV-3 cancer cells bind more ProCA22 compared to
MBD-MDA-231. These results indicated that the designed ProCA22 was able to target the cancer cells with high HER-2 expression level.

2.13. MR imaging of protein based contrast agents

We examined the in vivo performance of MRI of CD-1 mice (20-25g, N = 4) using a 4.7 T Varian MRI system with a dedicated rodent coil with modified parameters and variable pulse sequences. During MR scanning, the mice were anesthetized with 1.5% iso-flurane and kept warm with a heated pad. MR images were acquired by T₁- and T₂-weighted fast spin echo sequences (TR = 2 s, TE = 0.022 s, and ESP = 0.01 s) with field of view of 3×3 cm, matrix of 256×256, and slice thickness of 1 mm. The tumors were dissected after MRI experiments. Image J was used to quantitatively analyze the MRI images obtained. The regions of interest (ROI) were selected by circling the tumor sites. Then the signal intensities of the ROIs were calculated and compared. Six adjacent slides were selected to measure signal changes which were averaged to obtain statistically significant results.

Here we also introduced an optical imaging capability by conjugating a near-IR dye, Cy5.5 Mono Maleimide, a dye that produces an intense signal in the Near IR (NIR) region of the spectrum, to a Cystine residue at the C-terminal of the protein to facilitate imaging analyses. The Cy5.5 Mono Maleimide has a maximum absorbance at 674 nm and a maximum emission wavelength at 689 nm. The labeling yield is as high as 70%; the unlabeled free dye was separated from labeled proteins by dialysis in 10 mM HEPES buffer. Cy5.5 Mono Maleimide NIR imaging has high sensitivity and accompanied with the MR imaging to confirm the accurate targeting of selected biomarkers to cancer cells.
2.14. Immuno-histo-Chemistry (IHC) staining

To further verify the designed protein based contrast agent targeted to the HER-2 positive tumor, we carried out immunohistochemistry (IHC) staining using the antibody PAb with tissue slides made from the tumor samples and selected organs collected from the mice after MR and NIR imaging. Three different kinds of antibodies were applied during this process and then samples were taken images under the microscope based the vary fluorescence dye labeled on correlated antibodies.

2.15. Statistical Analysis

Data are presented as mean ± SEM. Parameters derived from the Hill equation and equations 3 were compared using a one-way analysis of variance with a Holm-Sidak multiple comparison as a post-hoc test. Statistical analysis was performed using SigmaStat 3.1 (Systat Software, Point Richmond, CA). Statistical significance was set at p<0.05.
3. **Ca\(^{2+}\) Binding Affinity and the Activation Profile are tuned by Site Specific Modification of Calmodulin**

CaM regulates the functions of many target proteins, including a number of ion channels in multimodal. For example CaM can facilitate or inhibit Ca\(_{\text{v}}\)2.1 depending on which lobe of CaM is Ca\(^{2+}\)-bound \(^{29}\). Similarly, RyR1 activation can be enhanced or inhibited depending of the Ca\(^{2+}\) saturation of the C-terminal lobe. Thus, by altering the Ca\(^{2+}\) affinity and thereby the Ca\(^{2+}\) saturation state of a specific lobe of CaM, it should be possible to selectively enhance (or reduce) a particular mode of CaM regulation of ion channels.

In this work, we tuned the activation profile of the RyR1 channel by introducing a point mutation into CaM (Asn97Asp) that generated a CaM variant which when bound to a putative RyR1 CaM-binding peptide specifically increased the Ca\(^{2+}\) affinity of CaM’s C-domain without altering the Ca\(^{2+}\) affinity of the N-domain or substantially altering the mode of interaction with the peptide. To identify this site as critical for the conversion of CaM from a channel activator to an inhibitor, CaM mutations were designed to increase the affinity of each individual CaM EF-hand Ca\(^{2+}\)-binding motif by increasing the number of acidic residues in Ca\(^{2+}\)-coordinating positions within each EF-hand. The Ca\(^{2+}\) affinity of each of CaM’s lobes was then determined by monitoring the Ca\(^{2+}\) dependence of the intrinsic phenylalanine and tryptophan fluorescence, which has previously been shown to reflect Ca\(^{2+}\) binding to sites I and II of CaM’s N-domain and sites III and IV of CaM’s C-domain, respectively \(^{57}\). The functional effects of these substitutions were then
determined by examining CaM modulation of Ca\(^{2+}\) activation of the RyR1 Ca\(^{2+}\) release channel.

3.1. Analysis of the coordinated binding of Ca\(^{2+}\) with CaM and the induced conformational changes

CaM is an important intracellular protein. There are two globular domains connected by a flexible linker in CaM. Each of the two domains contains a pair of well conserved canonical EF-hand calcium binding motifs which bind Ca\(^{2+}\) in a cooperative manner. The canonical EF-hand consists of a helix-loop-helix motif. Ca\(^{2+}\) is coordinated within the EF-hand by seven oxygen atoms from six amino acids, five of these residues are within the loop, and the sixth is located in the exiting helix. The coordinating residues can be designated as in a classical EF-hand loop by their positions in the linear sequence within the loop (1, 3, 5, 7, 9, and 12) and by their locations in the tertiary geometry of the coordinating site (±X, ±Y, and ±Z). The residues of the coordinating site form a pentagonal bipyramid where the Y- and Z-axis pairs align along the vertexes of a planar pentagon with the X axis pair lying perpendicular to the Y- and Z-axis plane. The residue at position 1 (+X) is a conserved aspartate and position 3 (+Y) is most frequently an aspartate or asparagines. The residue at position 9 (-X) does not directly coordinate Ca\(^{2+}\), rather the interaction is mediated by a bridging water molecule. The residue at position 12 (-Z) is a conserved bidentate glutamate. Unlike the other coordinating residues which chelate Ca\(^{2+}\) via side-chain oxygens, the residue at position 7 (-Y) coordinates Ca\(^{2+}\) via its backbone carbonyl oxygen. Residues 7 to 9 form a small
anti-parallel beta-sheet between the two coupled EF-hands within each domain (Figure 3.1).

The key factors contributing to the Ca\(^{2+}\)-binding affinity of EF-hand proteins, especially CaM, has been the subject of extensive work \(^{67, 68, 69, 70}\). Thus, CaM variants were designed based on considerations of site-specific calcium binding affinities, cooperativity between EF-hands within a domain; inter domain cooperativity and impact on CaM regulatory function. First, increasing the number of negatively charged residues from three to four within the EF-hand coordination sphere stabilizes the protein-Ca\(^{2+}\) complex. Increasing the number of charged residues beyond four may decrease the stability of the complex due to electrostatic repulsion between nearby similarly charged residues \(^{70}\). Four CaM variants, Thr26Asp, Asn60Asp, Asn97Asp, and Gln135Asp, were designed with one additional charged ligand residue (Asp) introduced into EF-hand loops I, II, III and IV (Figure 3.1), respectively. Site I of wild type CaM contains four acidic residues at coordination positions +X, +Y, and ±Z. The –Y and –X positions are occupied by threonines. Because the residue at the –X position contributes to Ca\(^{2+}\) coordination via water bridge, the –Y residue (Thr26) was replaced with aspartate. Site II contains four acidic residues located at coordinating positions ±X, +Y and –Z, an asparagine at +Z and a threonine at –Y. Given the fact that the most common residue at the +Z location is aspartate \(^{66}\); the asparagine at residue 60 was replaced with aspartate. Site III contains three acidic residues at +X, +Y, and –Z along with an asparagine at +Z, a tyrosine at –Y, and a serine at –X. Following the same reason as for site II, an aspartate was substituted for the asparagine at residue 97 (+Z). Site IV is similar to site me, containing four acidic residues at + X, + Y and ± Z, a glutamine at –Y and an asparagine at –X. Again, following the same logic as for site I, the glutamine at residue
135 (-Y) was replaced by an aspartate. Second, domain specific calcium binding affinity can be altered by changing the electrostatic interactions between coupled EF-hands within a domain 70. Changing residues within each Ca^{2+}-binding site can have significant effects on inter domain cooperativity 69. Finally, by tailoring the affinity of individual CaM Ca^{2+}-binding sites, Ca^{2+} loading of the target-specific, functionally important lobe of CaM can be manipulated. This strategy will allow CaM regulation of the biological activity of specific targets, in this case the activation profile of RyR1, to be tuned.
Figure 3.1. Sequence alignment of Rat Calmodulin in the presence of Ca\textsuperscript{2+}.

A) Ca\textsuperscript{2+} -CaM reconstructed using PyMol with the PDB file 3cln as a template. The location of each EF-hand point mutations (T26, N60, N97, and Q135; red sticks) used in my work (chapter 3) and with the aromatic residues phenylalanines -- F (residues 12, 16, 19, 65, and 68; orange sticks) and tyrosines --Y (residues 99 and 138; blue sticks) contributing to the CaM intrinsic fluorescence is indicated. B) Residues coordinating Ca\textsuperscript{2+} within the pentagonal bipyramid are in bold and their location in the EF-loop, geometric position and location in the primary sequence is indicated at the top of the figure. The substitutions to increase the number of acidic residues in each EF-hand are indicated in red italics.
3.2. Conformational analysis of CaM variants and peptide binding effects on the secondary structures

To determine the effect of the EF-hand charge mutations on the conformation of CaM in solution and when bound to the RyR1<sub>3614-3643</sub> peptide, the secondary structure of the CaM variants was examined using far UV circular dichroism (CD). Consistent with the substantial α-helical content of both apo- and Ca<sup>2+</sup>-loaded CaM, wild type CaM had large negative troughs at 208 and 222 nm in buffer containing 1 mM EGTA and in buffer containing 1 mM Ca<sup>2+</sup> (Figure 3.2.A). In EGTA containing media, CaM variants Thr<sub>26</sub>Asp, Asn<sub>60</sub>Asp, and Asn<sub>97</sub>Asp (Figure 3.2.B-D) exhibited a 30-34% decrease in ellipticity at 222 nm. In contrast, the Gln<sub>135</sub>Asp substitution resulted in only a 9% decrease in ellipticity (Figure 3.2.E). In Ca<sup>2+</sup>-containing buffer, substitution in Ca<sup>2+</sup>-binding sites I and III caused a 30-40% decrease in ellipticity, while substitution in sites II and IV had only small effects (2-8% decrease).

Upon addition of the RyR1<sub>3614-3643</sub> peptide to wild type CaM in EGTA containing buffer, there was 22% decrease in ellipticity at 222 nm attributable to CaM (Figure 3.2.A). In Ca<sup>2+</sup> containing buffer, addition of the peptide caused a 9% increase in the ellipticity of CaM. Thus, both the Ca<sup>2+</sup>-free and Ca<sup>2+</sup>-bound forms of wild type CaM interact with the RyR1<sub>3614-3643</sub> peptide. CaM variants Thr<sub>26</sub>Asp, Asn<sub>60</sub>Asp and Asn<sub>97</sub>Asp showed similar ellipticity changes (27-39% decrease) upon the addition of RyR1 peptide in EGTA containing media (Figure 3.2.B-D). Again, the Gln<sub>135</sub>Asp (loop IV) CaM variant differed and exhibited a 64% decrease in ellipticity under these conditions (Figure 3.2.E).
In Ca\textsuperscript{2+}-containing buffer, the addition of RyR1\textsubscript{3614-3643} peptide caused a 9% increase in the wild type CaM ellipticity at 222 nm (Figure 3.2.A). CaM variants Thr26Asp, Asn60Asp, and Asn7Asp all exhibited similar increases (7-10%) in ellipticity upon the addition of peptide in Ca\textsuperscript{2+} containing media (Fig 3.2.B-D). The Gln135Asp CaM variant was again the exception. Upon addition of the RyR1\textsubscript{3614-3643} peptide to Gln135Asp CaM in Ca\textsuperscript{2+} containing media, there was a 22% decrease in ellipticity (Fig 3.2.E).

These results, summarized in Figure 3.2.F and 3.2.G, suggest that the addition of a charged residue to EF-loops I, II or III causes only minor conformational changes to the apo- and Ca\textsuperscript{2+}-loaded forms of these CaM variants. Further, these CaMs undergo similar conformational changes upon binding to the RyR1\textsubscript{3614-3643} peptide. In contrast, the conformations of the apo- and Ca\textsuperscript{2+}-loaded forms of the Gln135Asp CaM variant upon RyR1\textsubscript{3614-3643} peptide binding are likely to be significantly different from those of the bound wild type CaM.
Figure 3.2. CD spectra of CaM and the variants in apo-and holo-form with and without RyR1 peptide.

(A: wt CaM; B: Thr26Asp CaM; C: Asn60Asp CaM; D: Asn97Asp CaM; E: Gln135Asp CaM). Ca$^{2+}$ (broken lines) or EGTA (solid lines) and with RyR1$_{3614-3643}$ (grey) or without RyR1$_{3614-3643}$ (black). F and G) Molar ellipticities of CaM variants as a fraction of wt CaM without peptide in (F) EGTA form without RyR1$_{3614-3643}$ (black bars) or with RyR1$_{3614-3643}$ (white bars) and (G) Ca$^{2+}$ without RyR1$_{3614-3643}$ (black bars) or with RyR1$_{3614-3643}$ (white bars).
3.3. Effects of charge variants on domain specific Ca\(^{2+}\) affinity

Lobe-specific Ca\(^{2+}\) binding affinities of the CaM variants were determined by monitoring intrinsic phenylalanine and tyrosine fluorescence changes during equilibrium Ca\(^{2+}\) titrations \(^{57,58}\). **Figure 3.1.A** shows the positions of the intrinsically fluorescent residues. Although three phenylalanines are located in the C-terminal lobe (C-domain) VanScyoc et al \(^{57}\) showed that these residues are non-reporting. Thus, the decrease in phenylalanine fluorescence intensity reflects the conformational changes in the N-terminal lobe (N-domain) of CaM as a result of Ca\(^{2+}\) binding to sites I and II. The increase in tyrosine fluorescence signal shown in **Figure 3.3.C and D** reflects the conformational changes in CaM’s C-domain as a result of Ca\(^{2+}\)-binding to CaM sites III and IV. The Ca\(^{2+}\)-dependence of phenylalanine and tyrosine fluorescence of wild type and mutant CaMs were fit with the Hill equation and are illustrated in **Figure 3.3.A-D**.

CaM Ca\(^{2+}\) dissociation constants (\(K_d\)) and Hill coefficients (\(n_H\)) as well as the free energy of Ca\(^{2+}\) binding (\(\Delta G\)) derived from these fits and are summarized in **Figure 3.3.E-H** and in **Table 3. 1**. As previously established \(^{24,71}\), the C-domain of CaM has a significantly higher Ca\(^{2+}\) affinity than the N-domain (C-domain \(K_d = 2.04 \pm 0.02 \mu M;\) N-domain \(K_d = 11.5 \pm 0.68 \mu M, p<0.05\)). Overall, the free energy of Ca\(^{2+}\) binding to the C-domain was \(-1\) kcal/mol lower than the free energy of binding to the N-domain (**Table 3.1**). The Thr26Asp mutation at -Y in EF-hand I significantly increased the Ca\(^{2+}\) affinity of both the N- and C- domains. In contrast, the Asn60Asp mutation at +Z in loop II had no significant effect on Ca\(^{2+}\) affinity of either the N- or C- domain of CaM.
3.1. Lobe-specific Ca\textsuperscript{2+} affinity of EF-hand CaM mutants.

<table>
<thead>
<tr>
<th>CaM Variant</th>
<th>N-domain</th>
<th>C-domain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( K_d (\mu M) )</td>
<td>( n_{\text{Hill}} )</td>
</tr>
<tr>
<td>Wild Type</td>
<td>11.5 ± 0.68</td>
<td>1.72 ± 0.08</td>
</tr>
<tr>
<td>Thr26Asp</td>
<td>8.8 ± 0.46*</td>
<td>1.48 ± 0.13</td>
</tr>
<tr>
<td>Asn60Asp</td>
<td>13.1 ± 0.25</td>
<td>1.70 ± 0.06</td>
</tr>
<tr>
<td>Asn97Asp</td>
<td>14.6 ± 0.25*</td>
<td>1.61 ± 0.05</td>
</tr>
<tr>
<td>Gln135Asp</td>
<td>8.2 ± 0.36*</td>
<td>1.05 ± 0.02*</td>
</tr>
<tr>
<td>wtCaM-RyR1\textsubscript{3614-3643}</td>
<td>2.5 ± 0.02</td>
<td>1.2 ± 0.02</td>
</tr>
<tr>
<td>Asn97Asp-RyR1\textsubscript{3614-3643}</td>
<td>2.4 ± 0.02</td>
<td>1.2 ± 0.02</td>
</tr>
</tbody>
</table>

Equilibrium Ca\textsuperscript{2+} titrations were performed as described under Experimental. Data are presented mean ± SEM, n=3. *Significantly different from Wild Type, \( p<0.05 \). # indicates the significantly different from wtCaM-RyR1\textsubscript{3614-3643}, \( p<0.05 \). \( \Delta G(\text{kcal/mol}) = -RT\ln K_a \).
Figure 3.3. Equilibrium $\text{Ca}^{2+}$ titrations of wild type and CaM mutants monitored by phenylalanine and tryrosine fluorescence.

A and B) $\text{Ca}^{2+}$-induced decrease in N-terminal Phe fluorescence. C and D) $\text{Ca}^{2+}$-induced increase in C-terminal Tyr fluorescence. A and C) wild type and N-terminal CaM variants. B and D) wild type and C-terminal CaM variants. Titration curves were fit by the Hill equation, wild type CaM (○), Thr26Asp CaM (●), Asn60Asp CaM (■), Asn97Asp CaM (▲), Gln135Asp CaM (▼). E) Comparison of N-domain $\text{Ca}^{2+}$ $K_d$ for CaM variants; F) Comparison of C-terminal $\text{Ca}^{2+}$ $K_d$ of CaM variants. G) N-terminal Hill coefficient, H) C-terminal Hill coefficient. Data are the mean ± SEM, $n = 3$. *Significantly different from wild type CaM, $p < 0.05$. 

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Mutations in the C-domain had more significant effects on the Ca\(^{2+}\) affinity than mutations in the N-domain. The Asn97Asp mutation significantly increased the C-domain Ca\(^{2+}\) affinity, significantly decreased the N-domain affinity and thus further increased the difference in affinity between the two lobes of CaM. In addition, the free energy of Ca\(^{2+}\) binding to the C-domain of this CaM was the lowest of any lobe of any variant. In contrast to all other substitutions studied, the Gln135Asp mutation significantly decreased the Ca\(^{2+}\) affinity of the domain in which it was located and increased the Ca\(^{2+}\) affinity of the opposing domain. This substitution narrowed the difference in Ca\(^{2+}\) affinity between the two lobes and reduced the free energy difference between N- and C-domains to \(\sim 0.4\) kcal/mol (compare to \(\sim 1.0\) kcal/mol for wild type). Further, the Gln135Asp mutation was the only substitution to significantly affect the cooperativity of Ca\(^{2+}\) binding, reducing the Hill coefficient for the N-domain (n\(_H\): wild type = 1.72 \(\pm\) 0.08; Gln135Asp = 1.05 \(\pm\) 0.02) and thus uncoupling the cooperativity between sites I and II within the N-domain. Interestingly, this mutation did not significantly alter the cooperativity between sites III and IV in the C-domain.

Of the six Ca\(^{2+}\) coordinating residues within each of the four EF-hands of mammalian CaM, the Asp at positions 1 and 3 and the Glu at position 12 are conserved (Figure 3.1). The conservation of these residues suggests they are critical for CaM function and indeed, replacing the charged Glu with an uncharged Gln or Ala at loop position 12 (-Z axis) in any of the EF-hands substantially decreased the Ca\(^{2+}\) affinity of the binding site \(^{72, 73}\). The identity of the residue at each of the remaining positions is variable. Reid and Hodges \(^{67}\) proposed the acid pair hypothesis to relate the Ca\(^{2+}\) affinity of EF-hands to the location of negatively charged residues in chelating positions. This hypothesis predicted that the highest Ca\(^{2+}\) affinity would occur in an individual EF-hand when acid pairs are located in the X- and Z-planes and that additional carboxylate
residues would decrease the affinity of the binding site due to electrostatic repulsion. However, additional factors contribute to EF-hand Ca\(^{2+}\) binding affinity, including the hydrophobicity of the loop and flanking residues \(^{74,75,76}\), the nature of the chelating residues within the loop \(^{77}\), and electrostatic interactions, especially those between charged liganding residues \(^{78,79,80}\).

Work with synthetic peptides and uncoupled EF-hands in intact CaM supported the hypothesis \(^{73,81}\). Black et al \(^{68}\) directly tested the acid-pair hypothesis in coupled EF-hands by monitoring the N-domain Ca\(^{2+}\) affinity of intact CaM via the Ca\(^{2+}\)-dependent change in fluorescence of a Trp which replaced a Phe at residue 19 and systematically adding or removing acidic residues in Ca\(^{2+}\) coordinating positions within EF-hands I or II. Their findings generally supported the acid pair hypothesis in that increasing the number of acid pairs within each hand from zero, to one and then to two, increased the affinity of N-domain Ca\(^{2+}\) binding sites. Adding an additional acidic residue to either EF-hand further increased Ca\(^{2+}\) affinity which then fell upon the completion of the third acid pair. Rather than introducing an additional mutation to monitor the concentration dependence of CaM Ca\(^{2+}\) binding we took advantage of the method of VanScyoc et al and determined the domain specific Ca\(^{2+}\) affinities by monitoring Tyr and Phe fluorescence within each domain (Figure 3.3) \(^{57}\). The acid pair hypothesis was insufficient to predict our results. In accordance with the hypothesis, increasing the number of acidic residues from 3 to 4 (site III, Asn97Asp) increased the Ca\(^{2+}\) affinity of the C-domain. However, rather than decreasing the domain Ca\(^{2+}\) affinity, further increases in the number of acidic residues had variable effects including increasing the N-domain Ca\(^{2+}\) affinity (Thr26Asp), having no effect on the N-domain Ca\(^{2+}\) affinity (Asn60Asp), and decreasing the C-domain Ca\(^{2+}\) affinity (Gln135Asp). Differences in the location of the acidic residues did not seem to account for their varying effects on the Ca\(^{2+}\) affinity. The Thr26Asp variant

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increased the Ca\(^{2+}\) affinity while the Gln135Asp variant had a decreased affinity despite the fact that both mutations added a negative charge at the \(-Y\) position and resulted in acidic residues at \(+X, \pm Y\) and \(\pm Z\).

These results can be explained in terms of a charge ligand-balanced model in which both the number of negatively charged ligand residues and the balanced electrostatic dentate-dentate repulsion by adjacent charged residues are major determinants of the Ca\(^{2+}\)-binding affinities of EF-loops in CaM \(^{70}\). In addition to acidic residues at positions 1, 3, 5 and 12, EF-hand I also has positively charged Lys residues at positions 2 and 11 which may reduce the electrostatic repulsion arising from closely situated carboxylates. Thus, the Thr26Asp substitution increased the net charge from -2 to -3 and increased the N-domain Ca\(^{2+}\) affinity. In contrast, site IV has an additional negative residue at position 11. Thus, the Gln135Asp substitution increases the net charge within this site from -5 to -6, increases the charge repulsion within the EF-hand and decreases C-domain Ca\(^{2+}\) affinity.

Two CaM variants we created (Th26Asp and Asn60Asp) were similar to those generated by Black et al \(^{68}\) (Phe19Trp/Thr26Asp and Phe19Trp/Asn60Asp). Both studies reported an increase in the N-domain Ca\(^{2+}\) affinity upon replacing Thr 26 with Asp. However, the results differ in regards to the Asn60Asp variant. We observed no change in the N-domain Ca\(^{2+}\) affinity of the Asn60Asp CaM while Black et al \(^{68}\) reported a significant increase. Although substitution of Trp for Phe19 provided for a highly sensitive measure of the Ca\(^{2+}\)-induced conformational changes in the N-lobe of CaM, the substitution may subtly perturb the structure of CaM, and thus have also altered the Ca\(^{2+}\) affinities of sites I and II.
3.4. Functional effects of CaM EF-hand mutations on RyR1 activity

RyR1 channel opening exhibits biphasic Ca\(^{2+}\) dependence. CaM shifts this activation profile to lower Ca\(^{2+}\) concentrations, enhancing channel opening at low Ca\(^{2+}\) concentration and inhibiting opening at higher concentrations. Based on the lack of channel inhibition by a Ca\(^{2+}\)-insensitive CaM mutant, the RyR1 activation at low Ca\(^{2+}\) is ascribed to the Ca\(^{2+}\)-free form of CaM and the inhibition is attributed to Ca\(^{2+}\) binding to CaM. Using domain-specific EF-hand knockdown mutations, Rodney et al.\(^ {82}\) and Fruen et al.\(^ {83}\) showed that Ca\(^{2+}\) binding to the C-domain of CaM is the switch to convert CaM from a RyR1 activator to a channel inhibitor. However, recently published work\(^ {84}\) conflicts with this view. Boschek et al.\(^ {84}\) compared the Ca\(^{2+}\) dependence of skeletal muscle SR [\(^{3}\)H]ryanodine binding with Ca\(^{2+}\)-induced change in signals from CaM that had been fluorescently labeled on either the N- or C-domain and bound to a putative RyR1 CaM-binding peptide. The Ca\(^{2+}\)-dependent conformational changes in the C-domain of CaM occurred in the same concentration range as the Ca\(^{2+}\) activation of SR vesicle ryanodine binding. Further, the Ca\(^{2+}\)-dependent conformational changes in the N-domain of CaM occurred in the same concentration range as the Ca\(^{2+}\) inhibition of SR vesicle [\(^{3}\)H]ryanodine binding. Therefore, the authors concluded that Ca\(^{2+}\) binding to CaM’s C-domain caused activation of RyR1 and Ca\(^{2+}\) binding to CaM’s N-domain resulted in channel closure. We used our CaM variants to determine whether Ca\(^{2+}\) binding to CaM’s N-domain or C-domain are the switch to convert CaM from a RyR1 activator to a channel inhibitor. We reasoned that increasing the Ca\(^{2+}\) affinity of the functionally important CaM domain, with no change in the affinity of the opposing domain, would lower the Ca\(^{2+}\) concentration at which CaM was converted from a channel activator to a channel inhibitor (switch-point).
In the media containing sub-micromolar Ca\(^{2+}\), CaM increases RyR1 channel opening; whereas in the media containing micromolar Ca\(^{2+}\), CaM plays an inhibitory role. Therefore, to determine whether our EF-hand site mutations altered the ability of CaM to regulate RyR1 we determined the CaM concentration dependence of SR vesicle \(^{3}\text{H}\)ryanodine binding in media containing either 100 nM or 700 μM Ca\(^{2+}\). In media containing 100 nM Ca\(^{2+}\) (Figure 3.4), wild type CaM increased ryanodine binding 12.9 ± 0.9% with an EC\(_{50}\) of 109.0 ± 18.4 nM and a Hill coefficient of 1.07 ± 0.17. All four CaM variants increased SR vesicle ryanodine binding to a similar extent as wild type CaM (Table 3.2). However, the site II mutation, Asn60Asp, decreased the EC\(_{50}\) for CaM activation (Table 3.2). None of the remaining mutations altered the CaM EC\(_{50}\) for SR vesicle \(^{3}\text{H}\)ryanodine binding and none of the mutations significantly altered the Hill coefficient.

In media containing 700 μM Ca\(^{2+}\), wild type CaM decreased SR vesicle ryanodine 16.7 ± 0.8% with an IC\(_{50}\) of 30.9 ± 3.0 nM and a Hill coefficient of 1.80 ± 0.28 (Figure 3.4, Table 3.2). Gln135Asp was the only mutation to significantly alter the efficiency of CaM inhibition. At saturating concentrations, inhibition by this mutant was only 59% of the inhibition induced by wild type CaM. The reduced efficacy of inhibition suggests that this variant interacts with RyR1 in a mode that differs from that of wild type CaM. Two variants, Thr26Asp and Gln135Asp increased the CaM IC\(_{50}\) (73.7 ± 10.0 and 57.8 ± 6.7 nM respectively). None of the mutations altered the Hill coefficient.
Table 3.2. Fit parameters for the Calmodulin concentration dependence of activation

<table>
<thead>
<tr>
<th>Calmodulin Variant</th>
<th>Extent of Activation (% $B_{max}$)</th>
<th>$n_a$</th>
<th>$EC_{50}$ (nM)</th>
<th>Extent of Inhibition (% $B_{max}$)</th>
<th>$n_i$</th>
<th>$IC_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type</td>
<td>12.9±0.9</td>
<td>1.07±0.17</td>
<td>109.0±18.4</td>
<td>16.7±0.8</td>
<td>1.80±0.28</td>
<td>30.9±3.0</td>
</tr>
<tr>
<td>Thr26Asp</td>
<td>15.5±1.0</td>
<td>0.97±0.16</td>
<td>141.6±29.2</td>
<td>18.0±1.2</td>
<td>1.80±0.42</td>
<td>73.7±10.0*</td>
</tr>
<tr>
<td>Asn60Asp</td>
<td>15.3±1.5</td>
<td>1.32±0.36</td>
<td>27.4±6.0*</td>
<td>17.3±1.5</td>
<td>1.48±0.37</td>
<td>15.9±2.9</td>
</tr>
<tr>
<td>Asn97Asp</td>
<td>11.3±1.4</td>
<td>1.27±0.41</td>
<td>75.1±20.4</td>
<td>15.2±1.4</td>
<td>1.88±0.53</td>
<td>39.6±7.0</td>
</tr>
<tr>
<td>Gln135Asp</td>
<td>16.4±1.1</td>
<td>1.14±0.18</td>
<td>95.6±14.8</td>
<td>9.8±0.7*</td>
<td>2.60±0.70</td>
<td>57.8±6.7*</td>
</tr>
</tbody>
</table>

SR vesicle [³H]ryanodine binding was performed as described under Experimental in media containing either 100 nM Ca²⁺ for activation or 700 μM Ca²⁺ for inhibition.

*Significantly different from Wild Type, $p < 0.05$; n = 6-13.
Figure 3.4. CaM concentration dependence of activation (A, B) and inhibition (C, D) of SR vesicle \(^{3}\text{H}\)ryanodine binding.

Ryanodine binding was performed as described under Experimental in media containing 100 nM Ca\(^{2+}\) (A, B) or 700 μM (C and D) and the indicated concentration of CaM; \(n = 6-13\). Lines are fits to the Hill equation. A) Comparison of activation by wild type CaM (○) with activation by N-terminal mutants Thr26Asp CaM (●) and Asn60Asp CaM (■). B) Comparison of activation by wild type CaM (○) with activation by C-terminal EF-hand mutants Asn97Asp (▲) and Glc135Asp (▼). C) Comparison of inhibition by wild type CaM (○) with inhibition by the N-terminal mutants Thr26Asp CaM (●) and Asn60Asp CaM (■). D) Comparison of inhibition by wild type CaM (○) with inhibition by C-terminal EF-hand mutants Asn97Asp (▲) and Glc135Asp (▼). Note that for comparison and clarity the wild type data in panels A and C are duplicated in panels B and D respectively.
To determine whether manipulating the site-specific Ca\textsuperscript{2+} affinity of CaM modifies the RyR1 activation profile, we next examined the effects of CaM variants on the Ca\textsuperscript{2+} dependence of SR vesicle [\textsuperscript{3}H]ryanodine binding (Figure 3.5, Table 3.3). In the absence of CaM, ryanodine binding exhibited the expected biphasic Ca\textsuperscript{2+} dependence (EC\textsubscript{50} = 1.69 ± 0.25 μM; IC\textsubscript{50} = 367.1 ± 53.5 μM). With Ca\textsuperscript{2+} as the sole channel regulator, SR vesicle [\textsuperscript{3}H]ryanodine binding reached 63.1 ± 2.1% of the estimated maximal binding. Including 2 μM wild type CaM in the incubation media significantly reduced the extent of Ca\textsuperscript{2+} activation of RyR1 to 39.4 ± 3.0% of maximal, significantly reduced the Ca\textsuperscript{2+} EC\textsubscript{50} to 0.23 ± 0.08 μM and reduced the Ca\textsuperscript{2+} IC\textsubscript{50} to 70.6 ± 25.6 μM. This resulted in a leftward shift in the Ca\textsuperscript{2+} dependence of RyR1 activation such that both channel activation and inhibition occurred at lower Ca\textsuperscript{2+} concentrations. The switch from RyR1 activator to inhibitor is attributed to Ca\textsuperscript{2+} binding to CaM, thus we define a Ca\textsuperscript{2+} switch-point as the Ca\textsuperscript{2+} concentration at which CaM is converted from a channel activator to an inhibitor. In practice, this point was the Ca\textsuperscript{2+} concentration at which the line from equation 3 fitted to the ryanodine binding data obtained in the presence of CaM intersected the curve fitted to the binding data obtained in the absence of CaM, i.e. the Ca\textsuperscript{2+} concentration at which the ratio of [\textsuperscript{3}H]ryanodine bound in media containing CaM to the [\textsuperscript{3}H]ryanodine bound in media lacking CaM was equal to one (Figure 3.5 C and D). Thus, the switch point for wild type CaM occurred at 3.8 ± 0.7 μM Ca\textsuperscript{2+} (arrows labeled 1 in Figure 3.5). Including 2 μM CaM variants in the binding media caused a similar leftward shift in the Ca\textsuperscript{2+} dependence of ryanodine binding significantly decreasing both the Ca\textsuperscript{2+} EC\textsubscript{50} and Ca\textsuperscript{2+} IC\textsubscript{50}. Further, mutations in CaM’s N-terminal Ca\textsuperscript{2+}-binding sites had no significant effect on CaM-induced decrease in Ca\textsuperscript{2+}-activated SR vesicle ryanodine binding. Thus, the Ca\textsuperscript{2+} dependence of ryanodine binding in media containing N-terminal CaM mutants virtually overlapped the Ca\textsuperscript{2+}-dependence of binding in media containing wild type CaM.
In contrast, mutations in CaM’s C-domain Ca\(^{2+}\)-binding sites had substantial effects on CaM modulation of RyR1 activation. When Asn97Asp, the site III CaM mutant, was included in the binding media the extent of Ca\(^{2+}\) activation was significantly reduced compared to wild type CaM (27.1 ± 1.7% vs 39.4 ± 3.0%). When compared to wild type CaM, this site III CaM variant switched from activating RyR1 to inhibiting the channel at a significantly lower Ca\(^{2+}\) concentration (1.2 ± 0.2 μM vs 3.8 ± 0.7 μM). However, the site IV mutation Gln135Asp increased the extent of Ca\(^{2+}\) activation to 77.0 ± 7.3% of maximal. The switch-point for this CaM variant was significantly increased to 38.2 ± 7.2 μM, approximately 10 fold higher that the switch-point for wild type CaM.

<table>
<thead>
<tr>
<th>Calmodulin Variant</th>
<th>(B_{\text{max}}) (pmol/mg)</th>
<th>(B_{0}) (% of (B_{\text{max}}))</th>
<th>(EC_{50}) (μM)</th>
<th>(IC_{50}) (μM)</th>
<th>Switch Point (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No CaM</td>
<td>8.04±1.01</td>
<td>63.1±2.1</td>
<td>1.69±0.25</td>
<td>367.1±53.5</td>
<td></td>
</tr>
<tr>
<td>Wild Type</td>
<td>8.04±1.01</td>
<td>39.4±3.0*</td>
<td>0.23±0.08*</td>
<td>70.6±25.6*</td>
<td>3.8±0.7</td>
</tr>
<tr>
<td>Thr26Asp</td>
<td>6.57±1.14</td>
<td>31.1±2.6*</td>
<td>0.12±0.05*</td>
<td>115.3±49.1*</td>
<td>2.5±0.4</td>
</tr>
<tr>
<td>Asn60Asp</td>
<td>7.10±1.53</td>
<td>34.6±3.4*</td>
<td>0.13±0.06*</td>
<td>93.9±47.9*</td>
<td>3.4±0.4</td>
</tr>
<tr>
<td>Asn97Asp</td>
<td>7.20±1.08</td>
<td>27.1±1.7*#</td>
<td>0.37±0.15*</td>
<td>119.7±36.6*</td>
<td>1.2±0.2#</td>
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<tr>
<td>Gln135Asp</td>
<td>6.79±1.69</td>
<td>77.0±7.3*#</td>
<td>0.53±0.19*</td>
<td>59.8±22.3*</td>
<td>38.2±7.2#</td>
</tr>
</tbody>
</table>

SR vesicle [\(^{3}\)H]ryanodine binding was performed as described under Experimental ± 2 μM calmodulin. *Significantly different from No CaM, p<0.05; #significantly different from wild type CaM, p<0.05; n=6-14.
Figure 3.5. Effects of wild type and EF-hand mutant CaM on the Ca\textsuperscript{2+} concentration dependence of SR vesicle [\textsuperscript{3}H]ryanodine binding.

A) Comparison of No CaM (○), wt CaM (□) with Thr26Asp CaM (●) and Asn60Asp CaM (■). Arrows indicate Ca\textsuperscript{2+} switch point for wild type CaM (1), Thr26Asp CaM (2), Asn60Asp CaM (3). B) Comparison of No CaM (○), wild type CaM (□) with Asn97Asp (▲) and Gln135Asp (▼). Arrows indicate Ca\textsuperscript{2+} switch point for wild type CaM (1), Asn97Asp CaM (4), Gln135Asp CaM (5). C and D) SR vesicle [\textsuperscript{3}H]ryanodine binding expressed as binding with one of the CaM variants as fraction of SR vesicle ryanodine binding without CaM. Symbols and numbers in C and D have the same meaning as in A and B respectively. For comparison and clarity the No CaM and wild type CaM data from A and C are duplicated in B and D respectively.
To verify the effects of our Asn97Asp mutant on the Ca\(^{2+}\)-dependence of RyR1 channel opening, we compared the effects of wild type CaM and Asn97Asp CaM on the open probability (P\(_o\)) of RyR1 channels incorporated into planar lipid bilayers. Based on a switch point for wild type CaM of 3.8 μM Ca\(^{2+}\), in recording media containing 4 μM Ca\(^{2+}\), the addition of wild type CaM should not reduce channel P\(_o\). In contrast, because Asn97Asp CaM switches from a channel activator to an inhibitor at 1.2 μM Ca\(^{2+}\), Asn97Asp should decrease channel P\(_o\). Indeed, this was observed (Figure 3.6). In the representative experiment in 7A, the addition of 3 μM wt CaM increased channel P\(_o\) from 0.032 to 0.058. Figure 3.6.B shows an experiment in which two channels incorporated into the bilayer simultaneously. The addition of 3 μM Asn97Asp CaM reduced the average P\(_o\) of the two channels from 0.028 to 0.017. Summary data is presented in Figure 3.6.C. In media containing 4 μM Ca\(^{2+}\), wtCaM did not significantly alter RyR1 P\(_o\) (No CaM = 0.040 ± 0.025, 3 μM wild type CaM = 0.033 ± 0.017). In contrast, the addition of Asn97Asp significantly reduced channel P\(_o\) (No CaM = 0.032 ± 0.013, 3 μM Asn97Asp CaM = 0.011 ± 0.004).
Figure 3.6. Effects of wild type and N97D CaM on single channel open probability.

RyR1 channels were incorporated into artificial bilayers and currents recorded as described under Experimental conditions. Panels A and B show representative experiments in which channel activity was recorded before and after adding 3 μM wt CaM (A) or 3 μM N97D CaM (B). Lines to the left of the figure indicate the closed current level; openings are upward. Panel C shows that the addition of wt CaM did not significantly alter channel $P_o$. *Significantly different from No CaM; $p < 0.05$. 
3.5. Conformational changes and interactions with peptide affect Ca\textsuperscript{2+} binding affinities to the two domains of CaM

Ca\textsuperscript{2+}-induced conformational changes to the two domains of CaM are coupled phenomena\textsuperscript{85,86}. Thus, changes in the Ca\textsuperscript{2+} affinity of either the N- or C-domain could cause a change in Ca\textsuperscript{2+} affinity of the opposite domain. However, the direction of affinity change of the opposite domain does not appear to be linked to the direction of affinity change of the mutated domain. Indeed, in the CaM variants under study the direction of change could be the same in both lobes (Thr26Asp) or in opposite directions with the Asn97Asp mutation increasing the C-domain Ca\textsuperscript{2+} affinity and decreasing the N-domain affinity while the Gln135Asp mutation had the opposite effects.

The decreased C-domain Ca\textsuperscript{2+} binding affinity of the site IV Gln135Asp mutation and the increased N-domain affinity further support the notion that EF-hand motif IV mutations often result in a large change of Ca\textsuperscript{2+} binding affinity, possibly due to alteration in inter- and intradomain interactions. Fefeu et al\textsuperscript{69} reported that similar to our Gln135Asp mutation, the Val136Gly mutation altered CaM's inter domain interaction, and substantially decreased the C-domain Ca\textsuperscript{2+} affinity while increasing the N-domain affinity. The near-by very conservative Asp133Glu substitution not only drastically decreased the site IV Ca\textsuperscript{2+} affinity but also decreased the affinity of the coupled site III within the C-domain\textsuperscript{73}. Such significant alterations in site IV Ca\textsuperscript{2+} affinity are likely due to the clustering of a large number of negatively charged residues in both liganding positions 1, 3, 5, and 12 and in non-liganding position 11. This EF-hand motif also lacks the positively charged residues (Lys) at position 2 in EF-hand III and at positions 2 and 11 in EF-hand I.
In wild type CaM, the difference in the Ca\textsuperscript{2+} affinity of the two domains is such that the C-domain Ca\textsuperscript{2+} binding sites may be nearly saturated while the N-domain binding sites are only partially filled. Under the conditions used here, the N-domain Ca\textsuperscript{2+} $K_d$ of wild type CaM was approximately 5.6 times higher than the C-domain $K_d$. Mutations in Ca\textsuperscript{2+} binding sites I and II had little effect on the relative difference in the domain Ca\textsuperscript{2+} affinities (N-domain $K_d$/C-domain $K_d$: Thr26Asp ~6.4; Asn60Asp ~5.7). Although the Thr26Asp CaM variant bound Ca\textsuperscript{2+} at lower concentrations than wild type CaM, because of the similar increases in the N- and C-domain Ca\textsuperscript{2+} affinity, the relative Ca\textsuperscript{2+} saturation of N- and C-domains was not significantly altered. In contrast, the C-domain mutation significantly altered the ratio of N- to C-domain saturation. The site III mutation, Asn97Asp, doubled the N-domain $K_d$/C-domain $K_d$ ratio to 11.8 while the site IV mutation, Gln135Asp reduced the ratio by half to ~2.1. Thus, based on the fits to the Hill equation shown in Table 3.1 and Figure 3.3, at a free Ca\textsuperscript{2+} concentration of 1 μM, the N-domain saturation of wild type, Asn97Asp and Gln135Asp CaM for Ca\textsuperscript{2+} would be ~1.5, 1.3 and 9.8% respectively. The C-domain saturation of these variants for Ca\textsuperscript{2+} would be 16.2, 36.5, and 6.0% respectively.

In this regard, the N-terminal variants were not informative as neither mutation changed the relative domain Ca\textsuperscript{2+} affinity and therefore did not alter the Ca\textsuperscript{2+} switch point. The C-terminal CaM variants were more useful. The Asn97Asp mutation decreased the C-domain $K_d$ and lowered the Ca\textsuperscript{2+} concentration at which CaM switched from activating RyR1 to inhibiting the channel. Conversely, the Gln135Asp mutation increased the C-terminal Ca\textsuperscript{2+} $K_d$ and increased the Ca\textsuperscript{2+} concentration at which CaM was converted from a channel activator to a channel inhibitor. Thus, the Ca\textsuperscript{2+} switch-point moved in the same
direction as the change in C-domain $K_d$. Further, there was an inverse relationship between the N-terminal Ca$^{2+}$ $K_d$ and the Ca$^{2+}$ concentration at which the switch-point occurred (see Gln135Asp in Tables 3.1 and 3.2). We attribute the effects of the Asn97Asp mutant on the RyR1 activation profile to alteration in the variant’s Ca$^{2+}$ affinity. The concentration dependences of CaM activation and inhibition by the Asn97Asp overlapped those of wild type CaM suggesting this variant binds RyR1 in a mode that is functionally indistinguishable from wild type CaM. However, interpretation of the Gln135Asp results is more complex. Although the concentration dependence of activation by the Gln135Asp variant was similar to wild type CaM, the extent of inhibition by saturating concentrations of the mutant was significantly less than wild type. Thus, the large shift in the Ca$^{2+}$ switch-point associated with this variant is the result of both the altered Ca$^{2+}$ affinity of the CaM and the blunted maximal extent of inhibition. Further, upon binding to the RyR1$_{3614-3643}$ peptide there was a decrease in the ellipticity at 222 nm of the Ca$^{2+}$-loaded form of this CaM mutant rather than an increase as seen in wild type CaM. Therefore, this mutant likely binds RyR1 in a mode that differs from wild type CaM. However, in spite of these caveats, the current results, particularly those regarding the Asn97Asp CaM variant, are consistent with the initial proposal that Ca$^{2+}$ binding to CaM’s C-terminal is the inhibitory switch. We are currently carrying out detailed structural studies to examine the binding modes of these CaM variants.

Binding to target molecules can significantly alter the CaM Ca$^{2+}$-affinity$^{37}$. Therefore, we determined the lobe-specific Ca$^{2+}$ affinities of wild type CaM and the mutant Asn97Asp when the protein was bound to a peptide from the putative RyR1 CaM-binding domain (RyR1$_{3614-3643}$)$^{35,88}$. Binding CaM to the RyR1$_{3614-3643}$ peptide
similarly increased the Ca\(^{2+}\) affinity of the N-domain for wild type and Asn97Asp CaM (\(K_d\) with the peptide/\(K_d\) without the peptide: wild type ~5; Asn97Asp ~6) (Figure 3.7.A and B). Peptide binding had only a small effect on the wild type C-domain Ca\(^{2+}\) affinity (\(K_d\) in absence of peptide/\(K_d\) in presence of peptide: wild type ~3) but dramatically increased the Asn97Asp affinity (\(K_d\) ratio: ~18). As a result in media containing 1 μM Ca\(^{2+}\), the N-domain saturation of wild type and Asn97Asp CaM was similar, ~25.0 and 25.9% respectively. In contrast, the C-domain saturation of these variants was different with 56.7 and 99.9% respectively. Thus by selectively and substantially increasing the Ca\(^{2+}\) saturation of the C-domain, we were able to specifically enhance the RyR1 inhibitory function of CaM.
Figure 3.7. Domain-specific Ca\(^{2+}\) binding affinities to the wt CaM – RyR\(_{13614-3643}\) complex and the N97D-CaM – RyR\(_{3614-3643}\) complex.

A) Ca\(^{2+}\)-induced decrease in N-terminal Phe fluorescence; B) Ca\(^{2+}\)-induced increase in C-terminal Tyr fluorescence were determined by equilibrium Ca\(^{2+}\) titration. RyR\(_{13614-3643}\) peptide was mixed with wild type CaM (○) or Asn97Asp CaM (●) in a 1 to 1 ratio. Ca\(^{2+}\) titration was monitored by \(\lambda_{ex} = 250\) nm, \(\lambda_{em} = 280\) nm (phenylalanine) for the N-domain and by \(\lambda_{ex} = 265\) nm, \(\lambda_{em} = 292\) nm (tyrosine; to minimize the contribution from Trp in the peptide) for the C-domain of CaM.
3.6. Summary and potentials

We engineered a series of mutant CaMs designed to individually increase the Ca\(^{2+}\) affinity of each of CaM’s EF-hands by increasing the number of acidic residues in Ca\(^{2+}\) chelating positions. Domain-specific Ca\(^{2+}\) affinities of each CaM variant were determined by equilibrium fluorescence titration. Mutations in sites I (Thr26Asp) or II (Asn60Asp) in CaM’s N-terminal domain had little effect on CaM Ca\(^{2+}\) affinity and regulation of RyR1. However, the site III mutation Asn97Asp increased the Ca\(^{2+}\) binding affinity of CaM’s C-terminal domain and caused CaM to inhibit RyR1 at a lower Ca\(^{2+}\) concentration than wild type CaM. Conversely, the site IV mutation Gln135Asp decreased the Ca\(^{2+}\) binding affinity of CaM’s C-terminal domain and caused CaM to inhibit RyR1 at higher Ca\(^{2+}\) concentrations. These results support the hypothesis that Ca\(^{2+}\) binding to CaM’s C-terminal acts as the switch converting CaM from a RyR1 activator to a channel inhibitor. These results further indicate that targeting CaM’s Ca\(^{2+}\) affinity may be a valid strategy to tune the activation profile of CaM-regulated ion channels.

A number of skeletal muscle diseases including malignant hyperthermia (MH) and Duchenne muscular dystrophy (DMD) are genetic disorders leading to impaired cellular Ca\(^{2+}\) regulations. Mutations in RyR1 account for the majority of MH cases\(^99\) and leaky RyR1 channel may contribute to DMD\(^90\). RyR1 channels from MH susceptible individuals exhibit an enhanced sensitivity to CaM activation\(^91,92\) and possibly a decreased sensitivity to Ca\(^{2+}\)-CaM inhibition\(^93\). Therefore, targeting these muscles with a CaM that will reduce channel opening at low Ca\(^{2+}\) may be of therapeutic benefit. Further, the
enhanced Ca\textsuperscript{2+}-CaM inhibition of RyR1 may reduce the resting Ca\textsuperscript{2+} leak through the channels in DMD muscle. Because CaM regulates numerous cellular signaling pathways, an obstacle to this strategy will be to specifically target RyR1. As the CaM Ca\textsuperscript{2+}-binding site that acts as the regulatory switch varies with the CaM binding partner, we have taken an initial step toward achieving that specificity by targeting the Ca\textsuperscript{2+}-affinity of a single EF-hand.
4. Binding mode determination of CaM- RyR1 complex using a peptide mode through NMR study

4.1. Introduction of current knowledge about CaM binding sites on the ryanodine receptor determined by various methods

As discussed in Chapter 1 and 3, the type 1 ryanodine receptor (RyR1) with a tetramer structure is one of the major CaM binding proteins in sarcoplasmic reticulum (SR) membranes isolated from skeletal muscle (Figure 1.4)\textsuperscript{94,95}. CaM shifts the Ca\textsuperscript{2+} dependence of RyR1 opening to lower Ca\textsuperscript{2+} concentrations, effectively increasing channel opening at sub-micromolar Ca\textsuperscript{2+} and inhibiting the channel in micromolar Ca\textsuperscript{2+} concentration\textsuperscript{43}. The switch from a channel activator to a channel inhibitor is due to Ca\textsuperscript{2+} binding to CaM as Ca\textsuperscript{2+} insensitive mutants of CaM activate but do not inhibit RyR1\textsuperscript{36}. The Ca\textsuperscript{2+}-induced conversion of CaM from a RyR1 activator to a channel inhibitor suggests that the RyR1 activation profile might be manipulated by tuning the CaM Ca\textsuperscript{2+} affinity. So far, there are three potential CaM binding sites reported. A critical region for CaM binding within the RyR1 primary sequence, (RyR1\textsubscript{3614-3643})\textsuperscript{36}, has been identified by Hamilton and co-workers\textsuperscript{35}. It has reported that a peptide derived from this region displays a high affinity for both apo- CaM and Ca\textsuperscript{2+}-CaM\textsuperscript{96}. More recently, a second noncontiguous region within RyR1, RyR1\textsubscript{1975-1999}, has also been implicated in CaM binding\textsuperscript{97}, and current models suggest that the CaM binding pocket is formed by neighboring subunits of tetrameric RyR1\textsuperscript{84}. Hamilton’s group also discovered that a fragment of RyR1 from amino acid 4064-4210 is able to fold to be an EF- hand lobe to
bind to two Ca\(^{2+}\) ions and result in conformational change through binding to Ca\(^{2+}\) similar as CaM\(^*\). Ikemoto and Gangopadhyay also confirmed that the interaction of the CaM-binding domain (Lys3614–Asn3643) with the Cys4114–Asn4142 region (a region included in the CaM-like domain) serves as an intrinsic regulator of the RyR1\(^\text{99}\). The intrinsic tryptophan fluorescence of the RyR1\(_{3614-3643}\) peptide in complex with CaM\(^\text{100}\) indicated that this peptide target promotes a substantial increase in CaM’s affinity for Ca\(^{2+}\), suggesting that only nanomolar Ca\(^{2+}\) concentrations were required to inhibit the channel. On the other hand, functional studies demonstrate that much higher Ca\(^{2+}\) concentrations (~1 \(\mu\)M) are required for the inhibition of full-length RyR1 channels\(^\text{101,36,34}\).

Our previous results (Chapter 3) indicated that the C – terminal domain of CaM exhibited more important role to inhibit the channel than the N- terminal. In addition, using a Ca\(^{2+}\)-sensitive fluorescent derivative of CaM to probe affinity of CaM to the peptide, we have shown that CaM retains a high affinity for both the RyR1\(_{3614-3643}\) peptide and full-length RyR1. The N97D- CaM- RyR1\(_{3614-3643}\) complex displayed the highest binding affinity to Ca\(^{2+}\) than wild type CaM\(^\text{102}\). Therefore, the particular structure and Ca\(^{2+}\) sensitivity of CaM when in complex with RyR1 remain undefined, which is the key information to resolve the potential roles of CaM as a regulatory channel subunit. The solution structures of both Ca\(^{2+}\)-free and Ca\(^{2+}\)-loaded forms were determined by NMR spectroscopy\(^\text{103,104}\). A high degree of mobility near the middle of the central helix region in CaM, from residue K77 through S81 was observed by studying the backbone dynamics of Ca\(^{2+}\)-saturated recombinant Drosophila CaM using \(^{15}\)N longitudinal and transverse relaxation experiments, combined with \(^{15}\)N\(^{(1}\)H\)) NOE measurements\(^\text{105}\). CaM binds to the target enzymes in various binding modes and the two domains of CaM may play distinguish roles in the regulation of these enzymes. CaM-dependent kinases are activated when Ca\(^{2+}\)-CaM binds to the auto inhibitory region of the target with
hydrophobic patches in each of its two lobes to contact two hydrophobic anchor residues located at “1-10,” “1-14,” or “1-16” spacing in the primary sequence of the kinase. Other targets, including ion channels such as the ryanodine receptor, interact with CaM both in the presence and absence of Ca\textsuperscript{2+}. Although it is well established that CaM modulates Ca\textsuperscript{2+} activation of RyR1, the precise structural interaction between CaM and RyR1 is poorly defined. Further, the role of CaM in depolarization-induced Ca\textsuperscript{2+} release from skeletal muscle has not been established. The precise mechanisms by which CaM regulates RyR1 remain unknown due mainly to three major obstacles. First, RyR1, like a number of other CaM targets, is a Ca\textsuperscript{2+} regulated protein. Thus, it is difficult to unambiguously distinguish between the effects of Ca\textsuperscript{2+} directly on the channel and the effects of Ca\textsuperscript{2+} mediated via CaM. Second, because of its massive size (>2,000 kDa) and membrane localization, the structure of RyRs is not known at the atomic level as it is for a number of other ion channels. Although the location of the CaM binding site on 3-dimensional reconstructions of RyR1 is known, the molecular nature of binding site is not understood. This necessitates an indirect approach combining structural studies of CaM binding to synthetic peptides or minidomains based on the sequence of the putative RyR1 CaM binding site and CaM mutants designed to test specific models of the functional interaction between CaM and RyR1. Third, CaM modulates Ca\textsuperscript{2+} activation of RyR1, however, in vivo, the primary mechanism by which RyR1 is activated via an allosteric coupling to the L-type Ca\textsuperscript{2+} channel in the transverse-tubule membrane, with Ca\textsuperscript{2+} activation possibly playing a secondary role. CaM modulation of this allosteric activation of RyR1 has not yet been demonstrated due to the difficulty of simultaneously manipulating concentration and type of CaM in the skeletal muscle triad and controlling the membrane potential of the transverse-tubule membrane. We have developed a
unique approach to manipulate the intracellular milieu of muscle fibers will simultaneously controlling the membrane potential of the transverse-tubules.

Based on these previous studies, several questions need to be addressed:

1. Does apo CaM have the same binding mode as the Ca\(^{2+}\) loaded CaM?
2. Whether RyR1\(_{1975-1999}\) bind to both apo and holo- CaM or not, and what is the binding ratio?
3. How to distinguish the two binding regions of RyR1 with CaM?

In this chapter, we use peptide model and high resolution NMR to reveal the interaction modes of CaM to RyR by addressing these questions. To further investigate effects of RyR1 binding on CaM structure and Ca\(^{2+}\) sensitivity, the changes of chemical shift of CaM as a function of peptide: complex ratio in the absence and presence of calcium were examined based on the NMR assignments provided by Dr. Hing Wong. We have also performed RDC studies to confirm the binding modes. We then discussed the potential interaction working model and created mini domains encompassing multiple CaM binding sites for CaM to test the working model. Using this system we will examine the effects of CaM on the depolarization-induced Ca\(^{2+}\) release, the physiological activation process.
4.2. NMR studies of interactions of CaM with RyR1\textsubscript{3614-3643} binding region using peptide model

Peptides were designed and synthesized from Anaspec, INC. CaM and its variants were bacterially expressed, \textsuperscript{15}N-labeled and purified using established methods as detailed in Chapter 2. All NMR data were collected at 600 MHz varian NMR at GSU at 37 °C in 10 mM Tris base buffer, pH 7.4. \textsuperscript{1}H-\textsuperscript{15}N HSQC data of holo form CaM were collected and processed through NMR-pipe. Most residues can be assigned for the Ca\textsuperscript{2+}-CaM and the CaM – peptide complex (Figure 4.1). The chemical shifts from the residues in both N- and C-terminal domains are observed as shown in Figure 4.1. All the chemical shifts for the residues of CaM were calculated in Figure 4.2. The chemical shift changes were analyzed with different ratios of protein / peptide. Most changes of chemical shifts occur at the titration point of 1 to 0.9 (Figure 4.2 C) which indicated that CaM interacts with RyR1\textsubscript{3614-3643} at a ratio of one to one. Almost no chemical shifts changes when the ratio increased from 1:0.9 to 1:1.2 (Figure 4.2 D). The chemical shifts chart was analyzed for each residue in CaM – RyR1\textsubscript{3614-3643} complex at a 1:1.2 interaction ratio (Figure 4.3). Significant chemical shifts were observed in both domains and especially in the EF – loop region, suggesting that both the N - and C – terminal domains contributed to the interaction of CaM with the peptide.

Table 4.1 summarized the residues with dramatic signal intensity changes for CaM - RyR1\textsubscript{3614-3643} peptide complex. Most of these residues are located in the EF – loop region, such as K21, T29, A102, and I130. Some are even in the binding pocket, such as T26, D64, N137, indicating that the conformational changes occurs in the whole CaM protein during interacting with the peptide.
Figure 4.1. HSQC spectra of $^{15}$N-Ca$^{2+}$-CaM and CaM-RyR1$_{3614-3643}$ complex.

HSQC spectra of $^{15}$N-wt CaM in the presence of 10 mM Ca$^{2+}$ (red), Ca$^{2+}$-CaM mixed with RyR1$_{3614-3643}$ peptides at a 1 to 1.2 ratio (blue). Residues were labeled based on their chemical shifts and references.
Chemical shift calculated from the HSQC experimental spectra for Ca\(^{2+}\)-CaM with the RyR1\(_{3614-3643}\) complex.

Chemical shift calculated from the HSQC experimental spectra for \(^{15}\)N-Ca\(^{2+}\)-CaM with the RyR1\(_{3614-3643}\) peptide at a gradually increasing ratio from 1:0.3 (A), 1:0.6 (B), 1:0.9 (C) until to 1:1.2 (D) which is the saturated point.
Figure 4.3. Chemical shift for Ca\textsuperscript{2+}-CaM - RyR\textsubscript{1\textsubscript{3614-3643}} complex with 1 to 1.2 ratios. Chemical shift changes of individual residues of \textsuperscript{15}N-Ca\textsuperscript{2+}-CaM with the RyR\textsubscript{1\textsubscript{3614-3643}} peptide at the ratio of 1 to 1.2. Four EF – hand binding loops were labeled in the figure to be compared their chemical shifts changes.
Table 4.1. HSQC spectra Signal intensity of selected residues of the $^{15}$N-Ca$^{2+}$-CaM with the RyR1$_{3614-3643}$ peptide complex

<table>
<thead>
<tr>
<th>Residues</th>
<th>Signal Intensity</th>
<th>Signal Intensity</th>
<th>Signal Intensity</th>
</tr>
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<tr>
<td>[RyR1$_{3614-3643}$]/[CaM]</td>
<td>0.3</td>
<td>0.6</td>
<td>0.9</td>
</tr>
<tr>
<td>N-terminal domain</td>
<td></td>
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</tr>
<tr>
<td>K13</td>
<td>n.d.$^a$</td>
<td>0.69</td>
<td>0</td>
</tr>
<tr>
<td>K21</td>
<td>6.25</td>
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<td>0</td>
</tr>
<tr>
<td>G25</td>
<td>n.d.</td>
<td>1.11</td>
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</tr>
<tr>
<td>T26</td>
<td>n.d.</td>
<td>0.93</td>
<td>0</td>
</tr>
<tr>
<td>T29</td>
<td>5.26</td>
<td>0.67</td>
<td>0</td>
</tr>
<tr>
<td>G33</td>
<td>4.76</td>
<td>0.69</td>
<td>0</td>
</tr>
<tr>
<td>D64</td>
<td>4.76</td>
<td>0.62</td>
<td>0</td>
</tr>
<tr>
<td>C-terminal domain</td>
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<td></td>
</tr>
<tr>
<td>K94</td>
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<td>1.48</td>
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</tr>
<tr>
<td>A102</td>
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</tr>
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<tr>
<td>I130</td>
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<td>A147</td>
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<td>0</td>
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<tr>
<td>K148</td>
<td>9.09</td>
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$n^a$d. stands for not determined
HSQC spectra of the $^{15}$N-apo-CaM with the RyR1$_{3614-3643}$ peptide complex were also collected on the 600 MHz spectrometer. The spectra of apo- CaM titrated with gradually increased ratio of RyR1$_{3614-3643}$ peptide were shown in Figure 4.4 A–D. The chemical shifts changes introduced by the addition of the peptide suggested that the apo – CaM interact with the RyR1$_{3614-3643}$ peptide at a one to one ratio, similar to the Ca$^{2+}$ - CaM. Figure 4.5 is the comparison of chemical shift changes in apo –CaM (blue) and holo –CaM (orange). The changes of chemical shifts were observed in both N- and C -terminal domains for the CaM- RyR1$_{3614-3643}$ complex in the present of Ca$^{2+}$. However, mainly C- terminal has obvious chemical shift changes for the complex in the present of EGTA. These results indicated that CaM interacts with the RyR1$_{3614-3643}$ peptide in different modes in the apo – and holo – forms. Furthermore, the conformations for the CaM- RyR1$_{3614-3643}$ complex are distinguished with and without Ca$^{2+}$. These NMR studies suggest that CaM interacts with the RyR1 peptide with both domains in the presence of calcium while with mainly C-terminal domain of the protein in the absence of calcium.
Figure 4.4. HSQC spectra of EGTA-CaM and CaM-RyR1_{3614-3643} complex.

HSQC spectra of $^{15}$N-wt CaM of 10 mM EGTA with gradually adding RyR1_{3614-3643} peptide as the ratio of 1:0.3 (A), 1:0.6 (B), 1:0.9 (C), and 1:1.2 (D).
Figure 4.5. Chemical shifts comparison of CaM - RyR1$_{3614-3643}$ complex on 600 MHz in the presence of Ca$^{2+}$ and EGTA.

Chemical shift changes of HSQC signals from selected residues of the $^{15}$N-Ca$^{2+}$-CaM-RyR1$_{3614-3643}$ complex (orange) and $^{15}$N-EGTA-CaM - RyR1$_{3614-3643}$ complex (blue) obtained on 600 MHz.
4.3. **RDC study of interactions of CaM with at RyR13614-3643 binding region using a peptide model**

Residual dipolar coupling (RDC) has been used to assist in homology modeling, determine relative domain orientations in multidomain proteins, and characterize molecular binding. Here, we used RDC to study the binding mode of the apoCaM-RyR13614-3643 complex using high resolution NMR at 800 and 800 MHz. First, the $^{1}H-^{15}N$ isotropic couplings of the complex are measured by TROSY-based J-modulation experiments. Second, the complex was put into Pf1 phage media, and the couplings were measured again. Then RDCs were obtained by subtracting the isotropic couplings from the values measured in the second experiment.

To assess the binding mode, we compared the measured RDCs with calculated values from structures deposited in the protein data bank. We selected structures in which CaM binds by wrapping around the peptide using both N- and C-terminal domains (2HQW, 1CDM, 1SY9, 1IQ5, and 2BCX), as well as binding in an extended conformation (2IX7, 1CFF, 1K93, and 1G4Y). We also compared the data with the structure of the CaM by itself, both apo (1CFC) and holo (3CLN) forms. We found that our data fit best with the crystal structure of a complex between apoCaM and the IQ motif of myosin V, especially if only the coordinates of the N-terminal domain was used in the calculation (Figure 4.6 and Table 4.2). This was not surprise because most of the complexes were loaded with calcium, therefore the helices were in the open conformation, and will not fit well with the closed conformation of apoCaM. Interestingly, the N-terminal domain of the CaM-anthrax exotoxin complex (1K93, Table 2) also fit well
with our data, because in the structure, the N-terminal domain was free of metal ion even though they grew the crystal with saturating amount of calcium \(^\text{18}\).

The C-terminal domain does not fit well with available structures, suggesting that our complex has unique binding mode. These RDC data revealed binding mode about apoCaM are consistent with the NMR chemical shift analysis in Figure 4.4. indicated that only the C-terminal tail of apoCaM bind to the peptide. On the other hand, in the structures found in the PDB, the whole C-terminal domain is involved in binding.

In summary, our RDC data suggested that apoCaM binds in the extended conformation with the RyR1\(_{3614-3643}\) peptide, the N-terminal domain of CaM remains unchanged while the C-terminal domain binds to the peptide in a unique fashion.
Figure 4.6. Calculated RDC of apo-CaM formed RyR1\textsubscript{3614-3643} complex fitted

Residues in the N- and C-terminal domains were fitted separately with reasonable Q values for both two domains.
Table 4.2. RDC fitted factor comparison of apo CaM-RyR1 complex with complexes of CaM with other enzymes.

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<th>Pdb</th>
<th>Target</th>
<th>Binding mode</th>
<th>Q-factor</th>
<th>R&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of RDC in fitting</th>
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<td>0.74</td>
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<td>N/A</td>
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</tr>
</tbody>
</table>
4.4. **NMR study of interactions of CaM with RyR1\textsubscript{1975-1999} binding region using a peptide model**

Since a second noncontiguous region within RyR1, RyR1\textsubscript{1975-1999}, has also been implicated in CaM binding as recently reported\textsuperscript{97}, we collected the HSQC data on the 600 MHz spectrometer to detect interactions between CaM and RyR1\textsubscript{1975-1999}. Similar to the RyR1\textsubscript{3614-3643} peptide, the RyR1\textsubscript{1975-1999} peptide also interacts with CaM at a one to one ratio (Figure 4.7). Most residues in the Ca\textsuperscript{2+} - CaM - RyR1\textsubscript{1975-1999} complex were able to be assigned based on the original assignment of Ca\textsuperscript{2+} - CaM\textsuperscript{103,104}. The chemical shifts changes for each residue of CaM in the complex was calculated and summarized in Figure 4.8. The chemical shifts changes were observed in both two domains of CaM and even in the flexible linker region. Some residues in the EF – loop displayed dramatic chemical shift change. It is possible that the whole protein was involved in the interaction with this peptide and introduced a significant conformational change. Chemical shift changes of individual residues of \textsuperscript{15}N-Ca\textsuperscript{2+}-CaM with the RyR1\textsubscript{1975-1999} peptide at the ratio of 1 to 1.2. Four EF – hand binding loops were labeled in Figure 4.8 in order to compare their chemical shifts changes.
Figure 4.7. HSQC spectra of Ca$^{2+}$ loaded CaM and CaM-RyR1$_{1975-1999}$ complex.

HSQC spectra of $^{15}$N-wt CaM in the presence of 10 mM Ca$^{2+}$ (red), Ca$^{2+}$-CaM mixed with RyR1$_{1975-1999}$ peptides at a 1 to 1.2 ratio (cyan). Residues were labeled based on their chemical shifts and references.
Figure 4.8. Chemical shifts CaM–RyR1\_1975-1999 complexes collected on 600 MHz.

Four EF loops were labeled out and the residues with significant chemical shift changes in both N- and C-terminal domains bigger than 0.05 were labeled.
4.5. **Summary of CaM- RyR1 complex formations in both apo and holo forms with a proposed working model**

CaM regulates Ca$^{2+}$ release channel RyR1 in a Ca$^{2+}$ dependent manner$^{112,113}$. As shown in **Figure 4.9**, one of the conserved CaM binding domains in the RyR1 is believed in the region of 3614-3643$^{114,35}$. In the apo form, CaM binds to site 2 (RyR$_{3614-3643}$) through the C-terminal domain. If site 1 (RyR$_{1975-1999}$) is close to site 2, the N-terminal domain may also bind to site 1. After loading with calcium, CaMs can bind to both sites 1 and 2 using both N- and C- terminal domains. Apo- and Ca$^{2+}$-CaM may themselves induce RyR1 states that favor channel opening and closing. Alternatively, an interaction between site 2 (RyR$_{13614-3643}$) and site 3 (RyR$_{4114-4142}$) may be required for channel opening and disrupting the interaction may close the channel$^{99}$. Thus, the Ca$^{2+}$-induced shift in CaM binding to RyR$_{13614-3643}$ may disrupt the RyR$_{13614-3643}$/RyR$_{4114-4142}$ interaction and close the channel.
Figure 4.9. Our designed working Models of CaM Regulation on RyR1 in three possible binding regions.

Symbols labeled 1, 2, and 3 represent three sites on RyR1 composed of sequences 1975-1999, 3614-3643, and 4114-4142 respectively. CaM molecule is in green. Black dots were used to represent as Ca^{2+} ions.
As shown in our working models of CaM regulation on RyR1 (Figure 4.9), three sites located at separate positions in the channel are the potential binding regions for CaM. Symbols labeled 1, 2, and 3 represent three sites on RyR1 composed of sequences 1975-1999, 3614-3643, and 4114-4142 respectively. Apo-CaM binds to site 2 (RyR_{3614-3643}) via the C-terminal (Figure 4.9.A top). If site 1 (RyR_{1975-1999}) and site 2 are in close proximity, it is also possible that apo-CaM may span the two sites (Figure 4.9.B top). However, our NMR data is more supportive to the first model. At this point we have no data suggesting that the N-terminal domain of apo-CaM can interact with RyR_{1975-1999}.

In the Ca^{2+}-bound state, CaMs may bind to sites 1 and 2 (Figure 4.9.A bottom). Or alternatively, CaM may span sites 1 and 2 (Figure 4.9.B bottom). Given that [^{35}S]CaM binding and cryo-EM studies reported a single CaM binding site per RyR1 monomer, the spanning model is more likely. Our NMR data cannot eliminate either model 1 or model 2. In the peptide approach, both N- and C-terminus of CaM bind to peptides 1 and 2. However, this may not occur with the intact RyR1 molecule. This will be further tested using min domain encompassing both binding sites (see next section). Site 3 is composed of RyR_{4114-4142}. In both Figures 4.9.A and B, an interaction between this RyR_{4114-4142} region and RyR_{3614-3643} is illustrated along with its disruption by Ca^{2+}-CaM.
If only one site is recognized by CaM, we expect that the chemical shifts of CaM will be changed at 1:1 binding ratio of the peptide/protein complex. The affinity of CaM for the RyR1 peptide and mini domain is expected to be similar. However, our preliminary results by NMR shown in Figures 4.1, 4.4, 4.7 have demonstrated that both peptides \textsuperscript{RyR1\textsubscript{3614-3643}} and \textsuperscript{RyR1\textsubscript{1975-1999}} interact with CaM in a 1 to 1 ratio. There is uncertainty about how the N-terminal domain of Ca\textsuperscript{2+}-CaM interacts with RyR1. According to model 1 (Figure 4.9A), the N-terminal binds to \textsuperscript{RyR1\textsubscript{1975-1999}}. On the other hand, the N-terminal may not bind to \textsuperscript{RyR1\textsubscript{1975-1999}} according to model 2 (Figure 4.9B). Similar to our NMR results, a recent crystal structure of Ca\textsuperscript{2+}-CaM/RyR1 peptide complex shows that both the N- and C-terminus are bound to \textsuperscript{RyR1\textsubscript{3614-3643}}. To clarify these controversies, we will pay special attention to any NOE interaction between the N-terminal domain of CaM and RyR1 peptides. If there is no interaction between CaM and \textsuperscript{RyR1\textsubscript{1975-1999}}, then the \textsuperscript{1H-15N} HSQC spectrum should show no change when we switch from \textsuperscript{RyR1\textsubscript{3614-3643}} to mini domains.
4.6. Create mini domain encompassing multiple CaM binding sites of RyR1

To further test the above possibilities of CaM interaction both models and the potential interactions of CaM binding to several regions simultaneously, we have created mini domain of RyR1 encompassing multiple CaM binding sites from RyR1 showing below. A mini domain encompassing multiple CaM binding sites from RyR1 was designed. Two peptide fragments RyR13614-3643 and RyR11975-1999, encompassing 3614-3643 and 1975-1999 have been synthesized and were shown to bind CaM by several spectroscopic methods. This mini domain contains a flexible linker GGSGG to connect two putative CaM binding sites. The length of the linker is designed to cover the distance between two lobes of CaM based on a report assuming they bind to two different regions of RyR1 (Figure 4.9). Cornea et al used FRET between CaM and FKBP to estimate these distances\textsuperscript{115}. When the FRET donor was on FKBP and the acceptor was on the N-terminal of CaM, the distance was 67±3 Å in 30 nM Ca\textsuperscript{2+} and 65±3 angstroms in 30 μM Ca\textsuperscript{2+}. When the acceptor was on CaM's C-lobe the distances were 91±9 Å in 30 nM Ca\textsuperscript{2+} and 84±5 Å in 30 μM Ca\textsuperscript{2+}. The distances between the N- and C-lobes were 24 Å in 30 nM Ca\textsuperscript{2+} and 19 Å in 30 μM Ca\textsuperscript{2+}. Two different orientations are designed since CaM-complexes were reported to have both parallel and antiparallel binding modes\textsuperscript{10,116}. Studying both peptide models and mini domains allows us to unambiguously reveal the possible binding modes (orientation, stoichiometry) and binding affinity in the absence and presence of calcium. The peptide fragments have been produced using solid phase peptide synthesis. The mini-domains will be expressed by bacterial and purified using our tag-less method. In addition mini domains allow the protein fragments to be heterolabeled at a relative low costs.
Sequence of RyR1 1975-1999

\text{Ac-SRYALLMRAFTMSAAETARRTREFR-NH2}

Sequence of RyR1 1975-1999

\text{Ac-DRYGILMKAFTMTAAETARRTREFR-NH2}

Minidomain

\text{DRYGILMKAFTMTAAETARRTREFRGGSGGKSKAVWHKLLSK}
\text{QRRRAVVACFRMTPLYN}

The mini domain containing two CaM binding sites in RyR1 was designed into a pGEX-2T vector with a GST tag. The protein was expressed using BL21 (DE3) system and purified by glutathione sepharose 4B beads. The purified protein was further analyzed by mass spectrum (Figure 4.10). Mass spec result demonstrates that the protein was well purified with a correct molecular weight of 7271.7 Da.
Figure 4.10. Maldi - Mass spectrum of RyR11975-1999&3614-3643 mini domains after purification.

The molecular weight of the mini domain is about 7 kDa which shown in the spectra as a main peak.
5. The activation and inhibition of Ryanodine receptor channels by CaM isolated half domains and CaM with the linker region deleted

5.1. Introduction

CaM is a well known intracellular protein that plays a significant role in regulating the biological functions of numerous enzymes in the cell. The two globular domains are connected by a flexible helical tether. Upon binding of Ca\(^{2+}\), the secondary structure of each domain is not significantly changed but the helices and the linker region undergoes a large conformation reorientation as shown by the 3-D structures of CaM (Figure 1A). The B, C helices and F, G helices in each domain move away from the hydrophobic core due to the binding of Ca\(^{2+}\), which results in the exposure of the core in each domain to the solvent and greater availability for target binding to other enzymes. In addition, CaM may adjust \([Ca^{2+}]_i\) via regulation of ion channel and pumps and differentiate between local and global \([Ca^{2+}]_i\) changes among its different domains. Persechini, A., et.al performed a additional mutation by deleting seven amino acid residues (DQLTEEQ, residues 2–8) in the N terminal domain of CaM and reported that no activity was observed in CaM-dependent activation of skeletal muscle myosin light chain kinase indicating that the first several residues in the N-terminal domain were essential for the function.

As discussed in Chapter 1 and 3, RYR1 functions as a calcium release channel in the sarcoplasmic reticulum, as well as a connection between the sarcoplasmic
reticulum and the transverse tubule. Mutations in the RYR1 gene are associated with malignant hyperthermia susceptibility, central core disease, and minicore myopathy with external ophthalmoplegia. Alternatively spliced transcripts encoding different isoforms have been described. The RyR1 channel opening exhibits biphasic Ca\(^{2+}\) dependence. CaM shifts this activation profile to lower Ca\(^{2+}\) concentrations, enhancing channel opening at low Ca\(^{2+}\) concentration and inhibiting opening at higher concentrations.

In vitro, RyR1 channel opening exhibits a biphasic Ca\(^{2+}\) dependence attributed to high- and low-affinity Ca\(^{2+}\) binding sites which when occupied will activate or inhibit channel opening respectively. CaM shifts the Ca\(^{2+}\) dependence of RyR1 opening to lower Ca\(^{2+}\) concentrations, effectively increasing channel opening at sub-micromolar Ca\(^{2+}\) and inhibiting the channel in micromolar Ca\(^{2+}\). The switch from a channel activator to a channel inhibitor is due to Ca\(^{2+}\) binding to CaM, as Ca\(^{2+}\)-insensitive mutants of CaM activate but do not inhibit RyR1. Hamilton’s group found that adding three amino acid in the N-terminal domain of Drosophila CaM increases the affinity for the RyR1, and noted that the N–terminus contributes greatly to the binding to RyR1, they also suggested that both N- and C-terminus were required for the channel function with C- terminus as the primary factor.

Based on the lack of channel inhibition by a Ca\(^{2+}\)-insensitive CaM mutant, the RyR1 activation at low Ca\(^{2+}\) concentrations is ascribed to the Ca\(^{2+}\)-free form of CaM while the inhibition is attributed to Ca\(^{2+}\) bound to CaM. However, the contribution of each structural component of CaM to the regulation of RyR activity is not clear yet.
In this chapter, we use domain variants of CaM, the N- and C- terminal domain, the mutant with five amino acids deleted in the flexible linker region to dissect the contributions of these structural components of CaM to the RyR1 regulation. We first performed conformational analysis of these CaM variants using Circular Dichroism spectra. Then the equilibrium $[^{3}	ext{H}]$ryanodine binding studies have been performed by Dr. E. Balog to determine the binding capability of these structure components of CaM using the same procedures described in Chapter 2. Based on the additional studies of metal binding affinities of these structural domains detailed in Chapter 6, we show that the two half terminus and the flexible linker of CaM play different roles in regulating the function of RyR1.
Table 5.1. Steady-state kinetic parameters for binding and activation of enzymes by CaMs with deletions in the N-terminal leader sequence\textsuperscript{118}.

<table>
<thead>
<tr>
<th></th>
<th>skMLCK</th>
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<th>smMLCK</th>
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<tr>
<td></td>
<td>$K_{\text{act}}$</td>
<td>$K_i$</td>
<td>$F_{\text{act}}$</td>
<td>$K_{\text{act}}$</td>
<td>$K_i$</td>
<td>$F_{\text{act}}$</td>
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</table>

$K_{\text{act}}$ is the concentration of CaM or engineered CaM required for half-maximal activation of enzyme activity. $K_i$ is the apparent dissociation constant based on competitive inhibition of CaM-dependent enzyme activity. $F_{\text{act}}$ is the maximal enzyme activity expressed relative to the level of activity achieved with 100 nM CaM.
Figure 5.1. Model for the interaction of apoCaM and Ca$^{2+}$-CaM with RYR1. $C_1$, $C_2$ and $N_1$, $N_2$ are the C and N lobe binding sites on RYR1, respectively. The red sphere represents the C lobe of apoCaM. The red square represents the C lobe of Ca$^{2+}$-CaM. The blue sphere represents the N lobe of apoCaM. The yellow sphere represents Ca$^{2+}$.
5.2. The structural analyses using NMR and CD spectra

![Diagram](image)

**Figure 5.2.** Structural models of CaM variants, the isolated N-terminal, C-terminal domain and five residues deleted in the linker region.

a). N- and C-terminal domain of CaM comprise the whole globular protein CaM in a direct way. b). The helical linker between the two domains of CaM with a calculated size around 8 Å. c). The two domain combined together without the linker region to form an incomplete protein analog of CaM.
Fig. 5.2 shows the CaM variants with different structural domains (see chapter 6 for additional information. Near UV (250 nm ~ 320 nm) and far UV (190 nm ~260 nm) CD spectra were collected on the Jasco-810 spectropolarimeter at room temperature using a Tris-KCl buffer (10 mM Tris pH 7.4, 100 mM KCl) in a 1 cm light-path quartz cuvette. All spectra are background subtracted and the average of at least 10 scans. All the protein concentrations are 10 µM in 10 mM Tris buffer with pH 7.4 was used for far UV and near UV CD, respectively.

Figures 5.3 D-F shows that both the isolated half domains and wt CaM have similar far UV CD spectra with only the difference being ellipticity intensities between the far UV (right figures) CD spectra of wt CaM and the isolated N- terminal and C- terminal domains of CaM. All the proteins have similar secondary structures with typical helix spectra peak at 222 and 208 nm.

To further detect the tertiary structure of these CaM mutants, we used near UV CD scanned from 310 to 260 nm. Since the near UV signals are from the aromatic residues (Tryptophan, Tyrosine and Phenylalanine), it is well confirmed that all the CD signals at 260 - 265 nm for N –terminus (Figure 5.3.B) are generated by Phenylalanine residues located at positions 12, 16, 19,65, 68. On the other hand, the CD signals at 280 nm for C –terminus (Figure 5.3.C) mainly originateed from the two Tyr residues (Y99 and Y138) as well as from the three Phe residues (F89, F92 and F141). The CD signals were the combination of these two domains as shown in Figure 5.3.A.
Figure 5.3. Near UV and far UV CD spectra of wt CaM and N-, C-terminal domains of CaM.

A). Near UV CD spectra of wtCaM. B). Near UV CD spectra of N-CaM. C). Near UV CD spectra of C-CaM. D). Far UV CD spectra of wtCaM. E). Far UV CD spectra of N-CaM. F). Far UV CD spectra of C-CaM.
5.3. **Equilibrium $[^3\text{H}]$ryanodine Binding**

5.3.1. **The procedures of measuring equilibrium $[^3\text{H}]$ ryanodine binding**

Skeletal muscle heavy sarcoplasmic reticulum (HSR) vesicles were prepared from porcine longissimus dorsi muscle as previously described$^{119}$. $[^3\text{H}]$Ryanodine binding was performed by incubating HSR vesicles (0.2 mg/ml) for 90 min at 37 °C in medium containing 150 mM KCl, 10 mM PIPES, ± wild-type or mutant CaM (see figure legends for details), pH 7.0, 100 nM $[^3\text{H}]$Ryanodine, 1 mM EGTA and CaCl$_2$ to obtain the indicated free Ca$^{2+}$ concentration$^{120}$. HSR vesicles were then collected on Whatman GF/B filters and washed with 8 ml of ice-cold 100 mM KCl buffer. Radioactivity retained by the filters was determined by liquid scintillation counting. Estimates of maximal $[^3\text{H}]$ryanodine binding capacity of each HSR vesicle preparation were determined in medium that in addition contained 500 mM KCl, 5 mM ATP, and 100 µM Ca$^{2+}$. Nonspecific binding was measured in the presence of 20 µM nonradioactive ryanodine. $[^3\text{H}]$Ryanodine binding is expressed as a percent of maximal $[^3\text{H}]$Ryanodine binding. $[^3\text{H}]$Ryanodine binds to the open RyR with high affinity and specificity and is therefore a sensitive indicator of channel activity$^{21}$. Due to the number of mutations examined, it was not feasible to include all CaM variants in the same assay run. However, variants were always assayed with a control, either wild type CaM in the concentration dependence experiments or no CaM and wild type CaM when examining the Ca$^{2+}$ concentration dependence. As there were no statistical differences between the subsets of control experiments, the control experiments were pooled.

The CaM concentration dependence of SR vesicle $[^3\text{H}]$Ryanodine binding was fit with a four parameter Hill equation. Data relating the dependence of SR vesicle
ryanodine binding to Ca\(^{2+}\) concentration were fit with equation 3, where \(B_\text{o}\) is the maximal \(\text{Ca}^{2+}\)-activated SR \[^3\text{H}\]Ryanodine binding, \(\text{EC}_{50}\) is the Ca\(^{2+}\) concentration required to achieve the half-maximal binding, and \(\text{IC}_{50}\) is the Ca\(^{2+}\) concentration required to half-inhibit SR vesicle \[^3\text{H}\]Ryanodine binding.

\[
[^3\text{H}]\text{RyanodineBound} = B_\text{o}\left(\frac{[\text{Ca}^{2+}]}{[\text{Ca}^{2+}] + \text{EC}_{50}}\right)\left(1 - \frac{[\text{Ca}^{2+}]}{[\text{Ca}^{2+}] + \text{IC}_{50}}\right) \quad \text{Equation 5.1}
\]

5.3.2. The results and discussion for the equilibrium \[^3\text{H}\]ryanodine binding

The activity of the isolated CaM half domains and deletion linker CaM were tested by \[^3\text{H}\] Ryanodine bound assay in both the low (Figure 5.4, 100 nM) \(\text{Ca}^{2+}\) and high (Figure 5.5, 700 \(\mu\text{M}\) ) \(\text{Ca}^{2+}\) conditions. From the results shown in Figure 5.4 and Figure 5.5, in 100 nM Ca (apo-CaM), it appears that the N-terminal half CaM activates RyR1 but this a lower affinity than seen in wild type CaM. The linker deletion CaM also appears to activate RyR1, but with a lower affinity and to a lesser extent than in wild type CaM. The C-terminal half-CaM does not appear to activate RyR1. In 700 \(\mu\text{M}\) Ca (Ca-CaM), the N-terminal half CaM inhibits with a lower affinity than wild type. The C-terminal and Deletion CaMs do not appear to inhibit RyR1 even at high concentrations (10 \(\mu\text{M}\)). From the summarized table on the right of both figures, we can see that wild type CaM provides the lowest \(\text{EC}_{50}\) with stronger activity and inhibition compared with its variants in both the low \(\text{Ca}^{2+}\) and high \(\text{Ca}^{2+}\) solvent.
Figure 5.4. The $[^3]$H Ryanodine bound assay of CaM and its mutants at lower Ca$^{2+}$ concentration.

$[^3]$Hryanodine bound assay of CaM mutants at lower Ca$^{2+}$ concentration:

(●) N-CaM, (○ in red) C-CaM, (○ in green) Del-CaM and wt CaM (○) in the 100 nM Ca$^{2+}$ (apo-CaM) condition. The extent of activation, the hill number and the EC$_{50}$ was shown in the table on the right.

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<th>Mean ±SEM</th>
<th>Extent of Activation (% maximal binding)</th>
<th>n$_{H}$</th>
<th>EC$_{50}$ (nM)</th>
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<td>1.08±0.14</td>
<td>46.8±6.1</td>
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<tr>
<td>N-CaM</td>
<td>20.2±1.9</td>
<td>1.35±0.29</td>
<td>1350±272</td>
</tr>
<tr>
<td>Del-CaM</td>
<td>12.0±1.2</td>
<td>1.43±0.038</td>
<td>1296±298</td>
</tr>
</tbody>
</table>

Bold indicates significantly different from wild type CaM p<0.05; One-Way ANOVA with a Holm-Sidak test vs Wild type as a post-hoc analysis. N=4
Figure 5.5. The \[^3\text{H}\] Ryanodine bound assay of CaM and its mutants at high Ca\(^{2+}\) concentration.

The \[^3\text{H}\] Ryanodine bound assay of CaM mutants at high Ca\(^{2+}\) concentration:

(●) N-CaM, (○ in red) C-CaM, (○ in green) Del-CaM and wt CaM (○) in the 700 µM Ca\(^{2+}\) (holo-CaM) condition. The extent of activation, the hill number and the EC\(_{50}\) was shown in the table on the right.
Using domain-specific EF-hand knockdown mutations, Rodney et al.\textsuperscript{82} and Fruen et al.\textsuperscript{83} showed that Ca$^{2+}$ binding to the C-domain of CaM is the switch to convert CaM from a RyR1 activator to a channel inhibitor. However, recently published work conflicts with this view. Boschek et al.\textsuperscript{84} compared the Ca$^{2+}$ dependence of skeletal muscle SR [$^{3}$H]ryanodine binding with Ca$^{2+}$-induced change in signals from CaM that had been fluorescently labeled on either the N- or C-domain and bound to a putative RyR1 CaM-binding peptide. Further, the Ca$^{2+}$-dependent conformational changes in the N-domain of CaM occurred in the same concentration range as the Ca$^{2+}$ inhibition of SR vesicle [$^{3}$H]ryanodine binding. Therefore, the authors concluded that Ca$^{2+}$ binding to CaM’s C-domain caused activation of RyR1 and Ca$^{2+}$ binding to CaM’s N-domain resulted in channel closure.

We used our CaM variants to determine the activation and inhibition of CaM fractions on RyR1. We reported that increasing the Ca$^{2+}$ affinity of the functionally important CaM domain, with no change in the affinity of the opposing domain, would lower the Ca$^{2+}$ concentration at which CaM was converted from a channel activator to a channel inhibitor (switch-point). The N-terminal variants were not informative as neither mutation changed the relative domain Ca$^{2+}$ affinity and therefore did not alter the Ca$^{2+}$ switch point, while the C-terminal CaM variants were more useful.\textsuperscript{102} The switch points for all the fraction mutants as wild type CaM overlapped those of wild type CaM (Figure 5.5) suggesting domain mutant of CaM binds RyR1 in a mode that is functionally indistinguishable from wild type CaM. We determined the CaM concentration dependence of the SR vesicle [$^{3}$H]ryanodine binding in media containing either 100 nM or 700 μM Ca$^{2+}$ to determine whether the fraction mutations of CaM altered the ability of CaM to regulate RyR1. The reduced efficacy of inhibition suggests that the variants interact with RyR1 in a mode that differs from that of wild type CaM (Figure 5.4).
The concentration dependences of CaM activation and inhibition by the N-terminal CaM (1-75) require higher concentration than wild type indicating a function change for the isolated domain. However, the C-terminal domain of CaM (76-148) can neither activate nor inhibit the RyR1 channel even though it has a stronger binding affinity to Ca$^{2+}$ than the N-terminal domain. In sub-micromolar Ca$^{2+}$ the linker deletion CaM (Δ76-81) activated RyR1 to a lesser extent and with a lower affinity than wild type CaM. All these results confirm that the flexible linker region between the N- and C-terminal domain plays an essential role for the interaction of CaM with its targeting enzymes. The function will be damaged or even eliminated when this linker is destroyed. In addition, it seems that the C–terminal domain of CaM encountered more functional effects compared to the N-terminal and Del-CaM. We are currently carrying out detailed structural studies to examine the binding modes of these CaM variants.
6. Metal Binding, Conformational properties and Activity of PDE Analysis by Structural Components of CaM

6.1. Introduction

The 148 residue protein CaM contains four canonic EF – hands with Ca$^{2+}$ binding loops denoted I-IV and helices A-H. Each EF-hand pair in CaM forms a globular domain, in which the two EF- hands are linked by a short antiparallel $\beta$ - sheet. The two domains are connected by a flexible helical tether $^{105}$. Upon Ca$^{2+}$ binding the secondary structure of each domain is not significantly changed but the helices and the linker region undergo a large conformation reorientation as shown by the 3-D structures of CaM. The B, C helices and F, G helices in each domain move away from the hydrophobic core due to Ca$^{2+}$ binding which results in increased solvent exposure of the hydrophobic core in each domain, thus preparing CaM for targeted binding to other enzymes. The tryptic fragments of CaM $^{121,122}$, have shown to be very similar to the corresponding domains of intact CaM in terms of 3D structures as well as Ca$^{2+}$ binding characteristics $^{123,25}$. Although the ‘central helix’ linker of CaM in complex withCa$^{2+}$ is $\alpha$-helical in the crystalline state, NMR relaxation data conclusively shows that this linker is nonhelical and highly flexible near its mid-point $^{105}$. The functional importance of this plasticity was highlighted by the structures of CaM in complex with target peptides. In these complexes, the two CaM domains come together and clamp around the helical target peptide $^{106,13,107}$. Clearly, the flexibility of the inter-domain linker plays a key role in allowing the two domains to come together and permitting rearrangement of the relative positions of the
two domains to fit a wide array of target sites. Persechini’s group has reported that contacts with residues Ser-81 to Glu-84 are not critical in the CaM target complexes through their study of CaM mutants with selectively deletion of these residues. In addition, Persechini, A. et al proposed that the bending of the central helix may also occur in the native CaM target complex as well as in the Del-CaM-target complex as shown in Figure 6.1. Metal binding was demonstrated to be essential for the activation of CaM to form complex. The properties of Ca\(^{2+}\) binding to CaM have been well studied since CaM is the most important Ca\(^{2+}\) binding regulatory protein. However, the role of the central helix in the formation of target proteins in different binding modes is not clear.

**Figure 6.1. Superposition of the lowest energy structures.**

*a*, the N-terminal domain (Thr5–Arg 74) and *b*, the C-terminal domain (Glu 82 – Thr 146) of Ca\(^{2+}\)–CaM, derived using three different starting structures: apo CaM (1F70 and 1F71), a parvalbumin-derived homology model and *Paramecium* Ca\(^{2+}\)–CaM (1EXR). The backbone r.m.s. deviation relative to the mean is 0.24 Å.
Ca²⁺/CaM-dependent cyclic nucleotide phosphodiesterase (PDE1) is one of the key enzymes involved in the complex interactions between the cyclic nucleotide and Ca²⁺ second messenger systems. The overall structure of PDE1 isoforms is well conserved, consisting of four domains; two CaM-binding domains, an inhibitory domain and a catalytic domain. Zhang et al have reported that only mutations in the N-terminal lobe of CaM affect the activity of CaM to PDE1 although there are eight methionines in the hydrophobic clefts of Ca²⁺-CaM that are required for the binding and activation of PDE1. Thus, it is not clear what the contribution of different structural components of Ca/CaM is to the overall activation of PDE.

In this study, we first evaluate the thermal stability of the CaM variants with different structural components using circular dichroism (Figure 6.3), we then investigate their calcium binding affinities and compared with that of w.t. We then use PDE as an example to reveal the contribution of these structural components of CaM in the enzymatic activity of PDE.

### 6.2. CD analyses of secondary structures and unfolded temperatures.

The melting temperatures of these mutants in the presence of both Ca²⁺ and EGTA were determined by detecting secondary structural changes observed at 222 nm in a temperature range from 4°C to 94°C. The unfolded temperatures ($T_m$) were determined by CD spectra. The $T_m$ of Del-CaM was 110.02 °C in the presence of 1 mM Ca²⁺, while it decreased to 40.6 °C in the presence of EGTA, which is almost three times less than
Ca$^{2+}$ form. This clearly demonstrated that Del-CaM has higher stability after binding to Ca$^{2+}$. We were unable to obtain the T$_m$ of the Ca$^{2+}$ form CaM due to the high stability of CaM in the presence of Ca$^{2+}$ making the T$_m$ higher than 100 °C. T$_m$ values were obtained by fitting the data with sigmoid function as shown in **Equation 6.1**

$$Y = m1 + (m' - m1) / (1 + \exp(x - m3))$$

**Equation 6.1**

In **Equation 6.1**, m1 is the value of the starting point of Y, m' is the value of the last point of Y. m2 = m' - m1. m3 is the middle value of X and m4 is the rate of Y to X (Y/X) when close to the middle point.
Figure 6.2. Melting temperature of CaM with five residues deleted in the flexible helical linker region.

A and B). CD spectra in the presence of Ca\(^{2+}\). C and D). CD spectra in the presence of EGTA. Figure B and D were fitted by sigmoid function.
Figure 6.3. Melting temperature of CaM and its variants in the presence of Ca$^{2+}$ and EGTA determined by temperature based CD spectra.

A). Melting temperatures of CaM structural components fitted by sigmoid function in the presence of Ca$^{2+}$. B). Melting temperatures of CaM structural components fitted by sigmoid function in the presence of EGTA.
Table 6.1. Unfolded temperature determined by CD spectroscopy.

<table>
<thead>
<tr>
<th></th>
<th>wt- CaM</th>
<th>Del-CaM</th>
<th>N-CaM</th>
<th>C-CaM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tm (°C)- apo</td>
<td>53.3 ± 2.3</td>
<td>39.5 ± 0.5</td>
<td>51.1 ± 3.0</td>
<td>44.0 ± 1.7</td>
</tr>
<tr>
<td>Tm (°C)- holo</td>
<td>N/A</td>
<td>N/A</td>
<td>62.5 ± 10.1</td>
<td>57.1 ± 1.5</td>
</tr>
</tbody>
</table>

6.3. Ca$^{2+}$ binding affinities of isolated half domain of CaM and CaM with the flexible linker region deleted

Domain specific titrations of the N- , C- terminal CaM and Del – CaM were carried out by monitoring the Phe signals in the N- terminal domain and the Tyr signals in the C – terminal domain using the same method as wild type CaM. Figure 6.4. and Table 6.1 summarize the domain specific Ca$^{2+}$ binding affinities of wild type CaM and the fractions. Both N- terminal ($K_d$ is ~ 6.43 µM) and C – terminal ($K_d$ is ~ 1.18 µM) isolated domains of CaM have almost two folds increase in Ca$^{2+}$ binding affinities compared to the wild type ($K_d$ is ~11.5 µM for N - terminal and 2.04 µM for C – terminal).

For the mutants with five residues deleted in the linker region (Del-CaM), the $K_d$ decreased in both N- ($K_d$ is ~ 6.38 µM) and C- domains ($K_d$ is ~ 1.38 µM) compared to the wild type. The hill numbers of this deletion mutant are different in N- terminal and C – terminal domain for the deletion mutant ($n_H = 1.4$ in the N –terminal domain of del-CaM, $n_H = 2.4$ in the C –terminal domain) indicating that the cooperativity had changed ($n_H = 1.7$ in the N –terminal domain of Del-CaM, $n_H = 2.0$ in the C –terminal domain in wild type CaM) due to the deletion of the central linker.
Figure 6.4. Ca$^{2+}$ binding affinities of CaM and its variants tested by domain specific fluorescence spectrometry.

Ca$^{2+}$ binding affinities of CaM and its variants were tested using fluorescence titration by domain specific method: wt-CaM (○), N-terminal domain (□), C-terminal domain of CaM (★) and CaM with the linker region deletion (○).
Lobe-specific Ca\textsuperscript{2+} binding affinities of the CaM variants were determined via monitoring intrinsic fluorescence changes during equilibrium calcium titrations\textsuperscript{57,58}. The Ca\textsuperscript{2+} dependence of phenylalanine and tyrosine fluorescence of wild type and mutant CaMs were fit with the Hill equation and are illustrated in Figure 6.4. CaM Ca\textsuperscript{2+} dissociation constants (K\textsubscript{d}) and Hill coefficients (n\textsubscript{Hill}) were derived from these fits and are summarized in Table 6.2. As previously established, C-terminal lobe Ca\textsuperscript{2+} binding occurred with a significantly higher affinity that the N-terminal Ca\textsuperscript{2+} binding in the compact CaM protein\textsuperscript{102}, the isolated C – terminal domain still exhibits more than 5 folds stronger affinity than isolated N –terminal domain. The Ca\textsuperscript{2+} binding affinity to both the isolated N – terminal domain and C –terminal domain nearly doubled compared to the compact protein. These results confirmed the cooperative Ca\textsuperscript{2+} binding properties between the two domains of CaM. The hill numbers of the two domains are close to one
instead of two in the wild type CaM. Interestingly, Del-CaM provided stronger binding affinities in both domains with the $K_d$ values similar to the isolated domains. This indicated that the helical linker probably plays a role for the binding to Ca$^{2+}$. The Hill numbers of this deletion mutant are close to one instead of two, as seen in the whole compact CaM or in the isolated N- terminal, while they are close to two in the C – terminal domain. This result indicates that the deletion of the central linker decreased the cooperativity of the Ca$^{2+}$ binding to the two domains of CaM.

6.4. Size determination of isolated half domain- CaM and the mutant deleting flexible linker by pulse - field- gradient diffusion NMR

Figure 6.5 shows several highly-simplified models for the formation of the whole CaM molecule from its N and C-terminal domains. In the first case (Figure 6.5a); it is assumed that the N and C-terminal domains are spherical with hydrodynamic radii of $\sim 16$ Å. They bind to each other to form a larger globular protein with a volume of $4\pi R^3/3$, which is equal to $2[4\pi(16)^3]/3$, where R is the radius of the resulting protein. In other words, the resulting larger protein will have a radius of 20.2 Å. This is 26% increase, closer to the value suggested from a study by Altieri et al.$^{129}$ However, CaM is a protein of an elongated shape, with a central helix connecting the two globular sub domains (Figure 6.5b). In this case, the radius of the molecule would be the average of the three perpendicular axes. We measured the length of the central helix from the pdb file 3cln, which is $\sim 8$ Å. Therefore, the radius of the longest axis of CaM would be $32 + 4 = 36$ Å; and the average radius of CaM would be equal to $(16 + 16 + 36)/3 = 22.7$ Å. This is very close to our measured hydrodynamic radius of CaM by NMR diffusion experiments. In the third case (Figure 6.5c); we considered that the individual sub
domains form an elongated molecule without the central linker. In this case, the average radius would be \((16 + 16 + 32)/3 = 21.3\, \text{Å}\), which is also close to our experimental result for the Ca\(^{2+}\) loaded form of Del-CaM.

\[ \text{(a)} \quad \text{N-} + \text{C-} \rightarrow \text{CaM} \]

\[ \text{(b)} \quad \text{N-} \quad \text{C-} \quad \text{8 Å} \]

\[ \text{(c)} \quad \text{N-} \quad \text{C-} \]

**Figure 6.5**. Structural models of CaM variants, the isolated N-terminal, C-terminal domain and five residues deleted in the linker region.

a). N- and C-terminal domain of CaM comprise the whole globular protein CaM in a direct way. b). The helical linker between the two domains of CaM with a calculated size around 8 Å. c). The two domain combined together without the linker region to form an incomplete protein analog of CaM.

The isolated N-, C-terminal domains of CaM and the linker deletion variant of CaM were expressed and purified using the same method as wild type CaM. NMR samples contained 0.23 mM proteins in 10% D\(_2\)O, 100 mM KCl, and 10 mM imidazole at
pH 6.5, with 10 mM Ca$^{2+}$ for Ca$^{2+}$-bound or 1 mM EGTA for Ca$^{2+}$-free sample. The intensities of the protein signals collected on 600 MHz Varian Inova spectrometer were integrated from the methylene and methyl region of ~2 ppm spectral width (-0.2 – 1.8 ppm). The integrate regions for each species were carefully selected to avoid or reduce interferences from buffer signals. The integrated intensities were further normalized to minimize the experimental errors from phase adjustment and baseline correction during the processing. The data of isolated half domain CaM, CaM with the linker region deleted in both the Ca$^{2+}$ loaded (Figure 6.6.A) and Ca$^{2+}$ free (Figure 6.6.B) forms were fitted by mono exponential equation

$$Y = \exp(-m1^* M0^2); m1 = 0.002 \quad \text{Equation 6.2}$$

The radii of each mutant were calculated based on the fitting results using the equations described in Chapter 2.
Figure 6.6. PFG diffusion data of CaM and its variants in the presence of Ca\(^{2+}\) and EGTA on 600 MHz magnetic field strength.

PFG- diffusion data of isolated half domain CaM, CaM with the linker region deleted in both the Ca\(^{2+}\) loaded (A) and Ca\(^{2+}\) free (B) forms. The collected data points were fitted by one exponential equation based on the gradient strength function with the relative intensity processed on Felix 98.
Table 6.3 summarized the diffusion data for the apo and holo forms of CaM and its variants. All of our diffusion data can be fitted by equation 2 with correlation coefficient ≥ 0.999. The hydrodynamic radius of Ca\textsuperscript{2+}-CaM was determined to be 22.2 ± 0.4 Å. This result is compared to the crystal structure of Ca\textsuperscript{2+}-CaM (3CLN)\textsuperscript{130}, which has the well known dumbbell shape. The long axis of the crystal structure has a diameter of 68 Å, while the two perpendicular short axes have diameters of 30 and 40 Å, respectively. This gives an average radius of 23 Å, which agrees well with our hydrodynamic radius.

The apo form of CaM has a hydrodynamic radius of 21.4 ± 0.4 Å. This is comparable to the solution structure\textsuperscript{103}, which has an average radius of 22 Å (diameters of the long axis and the two short axes are 67, 33, and 33 Å, respectively). Our results indicated that there is no significant difference in the size of the Ca\textsuperscript{2+} free and bound forms of CaM.

For N60D, N97D double mutant CaM, the hydrodynamic radii were calculated to be 22.2 and 22.5 Å for the apo and Ca\textsuperscript{2+}-bound forms, respectively. As expected, these values are close to those of wt-CaM since the mutations should not change the size of the molecule.

For the CaM mutant that has 5 residues deleted from its central linker (Del-CaM), the apo form has a radius of 22.4 Å, which is essentially the same as that of wt-CaM. This indicates that the central linker is very flexible in the apo form so that it can accommodate changes in its length. However, after adding calcium, the radius decreased to 21.0 Å. The hydrodynamic radii of the N and C-terminal domains (N-CaM and C-CaM, respectively) were measured to be ~16 Å. They are almost identical in size, independent of the presence of Ca\textsuperscript{2+}.

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Table 6.3. Summary of diffusion constants and hydrodynamic radii from PFG diffusion experiment of CaM variants

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Diffusion constant (× 10^7 cm^2/s)</th>
<th>Hydrodynamic radius (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apo</td>
<td>10.0 ± 0.2</td>
<td>21.4 ± 0.4</td>
</tr>
<tr>
<td>holo</td>
<td>9.6 ± 0.2</td>
<td>22.2 ± 0.4</td>
</tr>
<tr>
<td>C-CaM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apo</td>
<td>13.3 ± 0.1</td>
<td>16.1 ± 0.1</td>
</tr>
<tr>
<td>holo</td>
<td>12.9 ± 0.1</td>
<td>16.6 ± 0.2</td>
</tr>
<tr>
<td>N-CaM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apo</td>
<td>13.2 ± 0.2</td>
<td>16.2 ± 0.2</td>
</tr>
<tr>
<td>holo</td>
<td>13.2 ± 0.1</td>
<td>16.2 ± 0.1</td>
</tr>
<tr>
<td>Del-CaM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apo</td>
<td>9.5 ± 0.1</td>
<td>22.4 ± 0.2</td>
</tr>
<tr>
<td>holo</td>
<td>10.2 ± 0.2</td>
<td>21.0 ± 0.3</td>
</tr>
<tr>
<td>N60D, N97D CaM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>apo</td>
<td>9.6 ± 0.1</td>
<td>22.2 ± 0.3</td>
</tr>
<tr>
<td>holo</td>
<td>9.5 ± 0.1</td>
<td>22.5 ± 0.3</td>
</tr>
</tbody>
</table>
6.5. Biological function test of the isolated half domain- CaM and CaM with the deleted linker region by PDE assay.

Ca$^{2+}$/CaM- dependent cyclic nucleotide phosphodiesterase (PDE1) is one of the key enzymes involved in the complex interactions between the cyclic nucleotide and Ca$^{2+}$ second messenger systems. The overall structure of PDE1 isoforms is well conserved, consisting of four domains; two CaM-binding domains, an inhibitory domain and a catalytic domain$^{126,127}$. Zhang et al have reported that only mutations in the N-terminal lobe of CaM affect the activity of CaM to PDE1 although there are eight methionines in the hydrophobic clefts of Ca$^{2+}$-CaM that are required for the binding and activation of PDE1$^{128}$.

In our study, the functional effects of PDE on the substrate of mant-cGMP resulting from interactions with Ca$^{2+}$-CaM of various concentrations was studied through a time based fluorescence titration method. The EC$_{50}$ obtained through fitting the fluorescence data demonstrated the activation capabilities of wt CaM and its variants.

**Figure 6.7 and Table 6.4** summarize the time based fluorescence results which were fitted by the Hill equation. Wild type CaM activated PDE with an EC$_{50}$ of 7.1×10$^{-9}$ ± 0.02 M. The Del-CaM mutant exhibited lower activation capability than wtCaM with an EC$_{50}$ of 3.8 × 10$^{-7}$ ± 0.03M. However, the capability of activation for the N-terminal domain of CaM on PDE decreased with nearly a four-fold increase of the EC$_{50}$ (2.55× 10$^{-8}$ ± 0.03 M) compared to wt CaM (7.1× 10$^{-9}$ ± 0.03 M). Conversely, the C-terminal domain of CaM lost its activity by dramatically increasing the EC$_{50}$ to 3.3 ×10$^{-7}$ ± 0.06 M, which are more than 40 folds greater than the wtCaM.

Zhang et al found that although there are eight methionine residues within the hydrophobic clefts of Ca$^{2+}$-CaM required for the binding and activation of PDE1 in both
two lobes of bovine CaM), only mutations in the N-terminal domain of CaM affect the activity on PDE1. They hypothesized that the C-terminal lobe of CaM may serve to target CaM to PDE1, while the N-terminal lobe activates the enzyme. Mutating the glutamate cluster of residues 82-84 to lysines abolishes and greatly impairs its activation of NAD kinase and MLCK respectively, but has no effect on phosphodiesterase. On the other hand, deletion of residues 79-80 abolishes its activation of phosphodiesterase but has no effect on MLCK. Therefore, the central linker may play a role of selectivity towards different targets of CaM. Biological analysis in Yuan’s group suggested that it is the helical structure in CaM binding domain 1 of PDE induced by the insertion of the indole ring from Tryptophan residue that allows this domain to bind both lobes of CaM.

We further analyzed the functional effects on PDE of our three mutants: the isolated N-terminal (1-75), C-terminal (76-148) and the Del-CaM mutant (i.e., deletion of residues 81-85). The half domains of CaM were found to activate PDE at a reduced activity with higher EC50s compared to the wild type protein. The C-terminal domain of CaM has a 50 times higher EC50 with virtually complete loss of activity. However, the N-terminal domain decreased the functional activity less than four times. These results confirmed the functional cooperativity between the two domains of CaM and indicated the effect on the C-terminal domain due to the lack of the N-terminal domain is more apparent. On the other hand, the mutant with the flexible linker also decreased the activating capability with an EC50 ~50 times higher than wtCaM. This may be explained by the reason that only five residues were deleted and the remaining residues in the linker still maintained the activated function assay to PDE although the activity was reduced to a lower level compared to the wt CaM. However, the isolated domains completely lost the cooperativity and were not able to function separately.
Figure 6.7. PDE function assay of CaM and its variants through time based fluorescence spectroscopy on the PTI fluorometer.

Data were fitted by hill equation.
Table 6.4. Summary of PDE function assay for wt CaM and the related mutants.

<table>
<thead>
<tr>
<th></th>
<th>wt- CaM</th>
<th>Del-CaM</th>
<th>N-CaM</th>
<th>C-CaM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$EC_{50}(\times 10^{-8}M)$</td>
<td>0.71± 0.02</td>
<td>38.2± 0.03</td>
<td>2.5± 0.003</td>
<td>32.76± 0.06</td>
</tr>
</tbody>
</table>

6.6. Conclusions and significance

We engineered structural components of CaM by molecular cloning. Our analyses indicated that these mutants maintain their structures similar to the wtCaM. The half domains of CaM exhibited stronger binding affinities with Ca$^{2+}$ than what we observed in the compact wtCaM. These results indicated that Ca$^{2+}$ undergoes cooperative binding to the two domains in CaM. Domain specific fluorescence results showed that the C-terminus has higher Ca$^{2+}$ binding affinity than the N-terminus. Interestingly, the Del-CaM mutant also provided stronger Ca$^{2+}$ binding affinity than the wild type in both the N- and C-terminal domains. Tm calculated from analyses of CD spectra suggested that all these structural components decreased their stability compared with wild type CaM. In vitro PDE function assay was carried out through time based fluorescence spectroscopy. The activity of CaM on PDE was abolished for the Del-CaM and the activity was greatly decreased for the C-terminal domain. However, the N-terminus was able to maintain its activity with an $EC_{50}$ value close to that of wtCaM. These results suggested that the N-terminal domain of CaM may play the essential role for the activity on PDE while the C-terminal and the central linker region may contribute to the binding between CaM and PDE instead of function.
7. Engineering Protein Based Contrast Agent with Multiple Metal Binding Sites and HER-2 Targeting Capability

7.1. Protein based contrast agents

Magnetic resonance imaging (MRI) has unique advantages in capturing 3-dimensional images of living systems with high spatial resolution and without many limitations or problems associated with other methods, particularly limited imaging penetration of tissue/organs and ionized radiation\textsuperscript{134,135}. MRI has emerged as a leading diagnostic technique in clinical and preclinical settings \textsuperscript{136,137}. In addition, it allows for non-invasive and repetitive assessment of biological processes with the same living subjects at different time points, which significantly reduces the number of animals required and cost\textsuperscript{138}. However, the application of MRI to assess specific disease markers for diagnosis and monitoring drug effects has been severely hampered by the lack of contrast agents with desirable properties including high relaxivity, minimal or no toxicity, and optimized \textit{in vivo} retention time\textsuperscript{139,140}. For example, gadolinium-diethylenetriamine pentaacetic acid (Gd-DPTA)\textsuperscript{141,142}, a current clinically approved MRI contrast agent, has a low relaxivity value of \(~5\text{ mM}^{-1}\text{s}^{-1}\) and requires an injection dose of up to 0.1 mmol kg\textsuperscript{-1} body weight for clinical application in animals and humans\textsuperscript{143}. In addition, Gd-DPTA may not be suitable for some advanced \textit{in vivo} MRI applications due to a high glomerular filtration rate for this small chelator molecule\textsuperscript{144,145}. The non-covalent binding of small chelators to plasma proteins, such as albumin (MS-325)\textsuperscript{146}, greatly increases the relaxivity with a good MR angiography\textsuperscript{146-147}. There is a strong need to develop MRI
contrast agents with improved relaxivity, optimized pharmacokinetics, and application to molecular imaging\textsuperscript{138a}.

HER-2 is a tyrosine kinase growth factor receptor and belongs to the human epidermal growth factor receptor (EGFR) family. HER-2 was first discovered as a rat oncogene called neu, found in rat neuroblastoma in the late 1970’s. It is expressed in a number of normal tissues which probably plays a role in normal cell function, growth regulation and proliferation\textsuperscript{148,149}.

HER-2 is over expressed in many malignances, such as breast cancer, urinary bladder carcinomas, and carcinomas in the gastrointestinal tract, to promote the growth of cancer cells\textsuperscript{150,151,152}. Figure 7.1 (cited from the published work of Wang, SE et al, 2011) shows the sequence, structure and dynamic conformational changes of the HER-2 affibody molecule. HER-2 serves as a co-receptor with related members of the HER family of tyrosine kinase–associated growth factors\textsuperscript{153,154}. Acquired amplification of the HER2/neu gene on chromosome 17 in HER-2-positive breast cancer leads to marked overexpression of HER-2 on the cell surface\textsuperscript{155}, which alters normal signaling function\textsuperscript{156,157}. Several targeted drugs such as Trastuzumab, a humanized monoclonal antibody that binds to HER-2, have been developed and very effective in inhibiting tumor-cell growth through a variety of intracellular, and possibly extracellular, mechanisms\textsuperscript{158,159}.
Figure 7.1. Structure and dynamics of the Z-HER-2 affibody molecule. (A) Sequences of the Z domain and Z-HER-2. Gray, randomized positions in affibody libraries; yellow, selected side chains at Z-HER-2 interface; cyan, other residues at Z-HER-2 interface; black boxes, dynamic nonpolar side chains in core; red boxes, Asn and Gln side chains with alternating conformations. Insets above and below sequences show secondary structure and surface exposure, with darker blue shading reflecting less surface exposure. Residues for which backbone NMR resonances in Z-HER-2 are broadened by dynamics are in gray and ordered regions in orange (matching coloring in C–E). (B) Backbone traces of the Z-HER-2 structure ensemble. The blue/gray colors of residues 1 to 12 at the N terminus show two conformers obtained with unrestrained SA. (C) Z-HER-2 conformational exchange dynamics: orange, no backbone dynamics; gray, backbone dynamics; white spheres, methyl groups at the interface between helices 1 and 2 that undergo conformational dynamics; sticks, dynamic Asn and Gln side chains. (D) The alternative Z-HER2_alt structure with an intact helix 1. (E) Illustration of how dynamics (as in D) coincides with mutations at the HER2 binding surface (yellow sticks corresponding to yellow shading in A).
Figure 7.2. Tumor-promoting function of TGF-β in HER2-overexpressing cancer cells is mediated by TGF-β-driven autocrine and paracrine ErbB ligands\textsuperscript{160}. 

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While HER2 does not have nature ligand, several proteins such as antibody and affibody have been developed to interact with HER2 with high affinity. Affibody molecules are small proteins being developed by a Swedish biotechnology company, Affibody AB. The original affibody protein scaffold was designed based on the Z domain (the immunoglobulin G binding domain) of protein A \(^{161}\). Protein A is a protein localized on the surface of Staphylococcus aureus which protects the bacterium from the immune system of the host. A slightly modified pattern of domain B, also known as domain Z consisting of 58 amino acids, has been extensively used as an affinity purification reagent. Thirteen solvent-exposed amino acids localized on helix 1 and helix 2 of this domain Z construct an affibody library by random organization. These affibody molecules are designed to imitate monoclonal antibodies (mAbs) and are engineered to bind to a large number of target proteins or peptides with high affinity. Affibody molecules are used in biochemical research and are being developed as potential new biopharmaceutical drugs\(^{162,163}\). Affibody molecules have also been reported to maintain stability under conditions of high temperature and high pH, which makes them interesting as potential molecules for tumor targeting applications \(^{164,165}\). To date, affibody has been reported to successfully target to HER2 over-expressed in different cancer types using various imaging technique such as PET (Fig 7.3) and NIR with high sensitivity.

Yang lab has successfully developed protein based contrast agents (ProCA1) by designing a singly metal binding site into a scaffold protein CD2 \(^{166}\). By controlling correlation time and increase water numbers in the coordination shell, ProCA1 has significantly increased relaxivity (Chapter 9). Based on the tunable calcium binding affinity and multiple metal binding sites and our detailed studies on the structural and
dynamic properties of CaM and our success in design protein based contrast agent, in this chapter and next chapter, we report our effort in engineering CAM as a MRI contrast agents for molecular imaging. We first examined the metal binding affinity especially for Gd$^{3+}$ and selectivity over several physiological metal ions of several CaM variants (Table 1.1). We then examined the relaxation properties and water numbers of these variants. Serum stability and metal toxicity were also investigated. Further, we eliminate the endogenous biological function of CAM in regulation by Pegylation. Finally, we further developed them to be targeted MRI contrast agents to against HER by addition of affibody moiety and GRPR by addition of peptide targeting sequence. They have been successfully applied to in invivo imaging using MRI and NIR by conjugating with NIR dye.

Figure 7.3 PET images from affibody with its specific target peptide.
7.2. Determining the metal binding affinities of designed protein based contrast agent with Gd$^{3+}$

Gd$^{3+}$-binding affinity of ProCA2 series protein-based contrast agents was determined by a competition titration with Fura-6F (a metal ion indicator, Invitrogen Molecular Probes) applied as a Gd$^{3+}$ indicator. The fluorescence spectra of Fura-6F were obtained with a fluorescence spectrophotometer (Photon Technology International, Inc.) with a 10 mm path length quartz cell at 22 °C. Fura-6F excitation spectra were acquired at two wavelengths 340 and 380 nm with an emission wavelength at 510 nm. Gd$^{3+}$-binding affinity of Fura-6F, $K_d$, was first determined by a Gd$^{3+}$ titration with Gd$^{3+}$ buffer system of 1 mM nitrilotriacetic acid (NTA). Free Gd$^{3+}$ concentration was calculated with a NTA Gd$^{3+}$-binding affinity of $2.6 \times 10^{-12}$ M. Fura-6F was mixed with Gd$^{3+}$ in 1:1 ratio for a competition titration. The experiment was performed by a gradual addition of ProCA22. An apparent dissociation constant, $K_{app}$, was estimated by fitting the
fluorescence excitation intensity ratio of Fura-6F at 340 and 380 nm with different concentrations of CaM and designed ProCA22 concentrations as a 1:1 binding model.

Gd\textsuperscript{3+}-binding affinity of ProCAs, $K_{d2}$, was calculated with the following equation:

$$ K_{d2} = K_{app} \cdot \frac{K_{d1}}{K_{d1} + [Fura-6F]_T} \quad \text{Equation 7.1} $$

Fluorescence dye competition experiments (Figure 7.5. a and b) were carried out to estimate the Gd\textsuperscript{3+} binding affinities with designed protein based contrast agents. The apparent binding constant $K_{app}$ was obtained by fitting the fluorescence signal intensity ratio at 340 nm and 380 nm (Figure 7.5.b). The dissociation constant $K_d$ was further calculated based on the Equation 7.1. The results indicated that protein based contrast agents had strong binding affinities to Gd\textsuperscript{3+} with a small dissociation constant $K_d$ in the range of $10^{-13}$ M. In addition, ProCA22 also exhibits high metal selectivity as determined by comparing the binding affinities between vary metals. The binding affinity of CaM to Ca\textsuperscript{2+} has been well studied for quite long time. The N- and C- domain of CaM have different binding affinities to Ca\textsuperscript{2+}, but both are in the µM range. The modified ProCA had similar binding affinity as wt CaM based on our experimental data. These data clearly showed that the designed ProCA22 has a strong binding affinity to Gd\textsuperscript{3+} with a reasonable metal selectivity to other metal ions, for example Ca\textsuperscript{2+}. 
Figure 7.5. Gd\textsuperscript{3+} binding affinity determination by a dye competition method.

Fura-6F with two excitation wavelengths at 340 nm and 380 nm and emission wavelength at 510 nm was selected to compete with ProCA22. The $K_d$ for Gd\textsuperscript{3+} to Fura-6F is $8.4 \times 10^{-12}$ M. The $K_{app}$ of Gd\textsuperscript{3+} with wt CaM and modified ProCA22 are $\sim 1.2 \times 10^{-6}$ M and the calculated $K_d$ are $\sim 5 \times 10^{-13}$ M.

7.3. Water coordination number measurement of the designed protein based contrast agent by Tb\textsuperscript{3+} lifetime luminescence

Wild type CaM and ProCA22 samples were initially prepared in H\textsubscript{2}O buffer system solutions, and then replaced with D\textsubscript{2}O by lyophilizing the samples and redissolving them in D\textsubscript{2}O. This procedure was repeated at least three times. Tb\textsuperscript{3+}-luminescent decay in both H\textsubscript{2}O and D\textsubscript{2}O were measured to determine the number of water ligands coordinated to the Gd\textsuperscript{3+}-ProCA22 complex. The Tb\textsuperscript{3+} excited-state lifetime was measured using a fluorescence spectrophotometer (Photon Technology International, Inc.) with a 10 mm path length quartz cuvet at room temperature (22 °C).
Following excitation at 265 nm with a XenoFlash lamp (Photon Technology International, Inc.), in a time based decay fluorescence experiment, Tb$^{3+}$ emission was monitored at 545 nm in both H$_2$O and D$_2$O systems. The luminescence decay lifetime was obtained by fitting the acquired data with a mono exponential decay function. The water number coordinated to the Tb$^{3+}$-ProCA22 complex was then obtained by fitting the acquired $\Delta k_{obs}$ value to the standard curve.

The lifetime of free Tb$^{3+}$ in H$_2$O and D$_2$O were found to be 410 and 2796 µs, respectively. The formation of the Tb$^{3+}$-wt CaM and ProCA22 in H$_2$O significantly increases Tb$^{3+}$ lifetime to 821 µs and 798 µs respectively. Tb$^{3+}$ life time values of wtCaM and ProCA22 were 1653 and 1606 µs respectively in D$_2$O, suggesting a hydration number of 1.6 and 1.8 (Figure 7.6.).
Determine the water coordination number of ProCA22 by measuring Tb$^{3+}$ life time luminescence.

Tb$^{3+}$ luminescence decay was measured in both H$_2$O and D$_2$O. Tb$^{3+}$ emission was monitored at 545 nm in both H$_2$O and D$_2$O systems with excitation at 265 nm. The water number coordinated to the Tb$^{3+}$-ProCA22 complex was obtained by fitting the acquired $\Delta k_{\text{obs}}$ value to the standard curve.
7.4.  *In vitro* relaxivity of the designed protein based MRI contrast agents

Relaxation times \((T_1\) and \(T_2)\) of various protein-based contrast agents were determined at 1.41 T using a 60 mq Bruker Mini Spec relaxometer. The lattice relaxation time \(T_1\) of ProCAs was determined at 1.41 T by inversion recovery pulse sequence and the data were fitted by Equation 2.9 in Chapter 2. The transverse relaxation time \(T_2\) of ProCAs was determined through \(t_2_{\text{cp.mb}}\) pulse sequence which is the Carr-Purcell-Meiboom-Gill (CPMG) spin echo method and the data were fitted by Equation 2.10 in Chapter 2. The protein based contrast agent samples (200 μL) with different concentrations were placed in matched glass tubes. The tubes were kept into water bath at 37°C for 10 min before measurement. \(r_1\) and \(r_2\) values were calculated based on Equations 2.11 and 2.12 in Chapter 2, where \(T_{1s}\) and \(T_{2s}\) are relaxation times with contrast agent and \(T_{1c}\) and \(T_{2c}\) are relaxation times without contrast agent. \(C\) is the concentration of contrast agent in the mM units.

The designed protein based contrast agent exhibited high relaxivity in both \(T_1\) and \(T_2\) relaxation test (*Figure 7.7. A - D*). Relaxivity of the modified protein was similar to that of the wt CaM. However, **Table 7.1**, shows that the \(r_1\) of ProCA22 (~50 mM\(^{-1}\)●S\(^{-1}\)) is almost 20- folds higher than Gd-DTPA (~ 3 mM\(^{-1}\)●S\(^{-1}\)). The relaxivity was even higher for ProCA22 (~70 mM\(^{-1}\)●S\(^{-1}\)) compared with Gd-DTPA (~ 3.4 mM\(^{-1}\)●S\(^{-1}\)). Since relaxivity is one of the most important factors for designing contrast agent, the high relaxivity is one of the potential advantages of our designed ProCAs, which indicated that it is possible that we can obtain significant MR images using lower doses compared to the commercial agents.
Figure 7.7. Relaxivity measured in 1.41 T Mini Spec relaxometer in 10 mM Tris buffer of pH 7.4 at 37 °C.

T₁ relaxation time was measured using an inversion recovery pulse sequence (A) and T₂ relaxaton time was measured by cp_mb pulse sequence (B). Concentration dependent time experiments were applied to determine the relaxivities r₁(C) and r₂(D).
Table 7.1. Relaxivities $r_1$ and $r_2$ of contrast agents determined by concentration dependent measurement at 37°C on the magnetic field strength of 1.41 T.

<table>
<thead>
<tr>
<th>Contrast agents</th>
<th>$r_1$ (mM•s⁻¹)</th>
<th>$r_2$ (mM•s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gd-DTPA</td>
<td>3.8</td>
<td>4.5</td>
</tr>
<tr>
<td>Wt CaM</td>
<td>53.9</td>
<td>76.4</td>
</tr>
<tr>
<td>ProCA22</td>
<td>50.0</td>
<td>67.3</td>
</tr>
</tbody>
</table>

7.5. Serum stability and PDE function test of ProCA22

Given that CaM is an important intra cellular protein and that it can modulate functions of a number of enzymes as well as regulate various channels, it is necessary to make sure that the designed protein-based contrast agent is no longer capable of functioning as wt CaM. We carried out phosphodiesterase (PDE) assay to test the function of ProCA22.

The phosphodiesterase (PDE) functional assay was conducted in 10 mM MOPS, 100 mM KCl buffer at pH 7.4. The PDE enzyme and the substrate 2'-methylanthraniloyl-cGMP (Mant-c-GMP) were purchased from Sigma Company. The experiment was completed using the PTI Fluorometer by choosing the time base acquisition at room temperature. The excitation wavelength of the labeled substrate is 282 nm and the emission wavelength is 442 nm.
Figure 7.8 A and B shows that the time-based fluorescence signal of Mant-c-GMP decreased with the addition of wt CaM, while the slope increased. However, almost no signal change was observed from the fluorescence spectra with the addition of ProCA22. These results indicated that ProCA22 has lost the ability to activate cGMP due to modification by PEGylation and insertion of targeting peptide of affibody. The wt CaM has EC$_{50}$ value about $10^{-8}$ M (A), while the ProCA22 almost lost the activity.

Figure 7.8 Phosphodiesterase (PDE) assay on time based fluorescence. Protein samples were prepared in the buffer containing 10 mM MOPS + 100 mM KCl with 200 µM EGTA + 10 mM CaCl$_2$. The final concentration of PDE is 0.25 µM, using Mant-c-GMP as a substrate.
7.6. Cell targeting test of the designed contrast agent grafting with cancer targeting peptide

Two human cancer cell lines, SKOV-3 and MDA-MB-231 were selected to examine whether the designed ProCA22 can target to cancer cells. SKOV-3 is an ovarian cancer cell line with estimated $3 \times 10^6$ HER2/cell. MDA-MB-231 is a breast cancer cell line with modest HER2 levels ($\sim 3 \times 10^4$ HER-2 / cell). Binding of the Gd-ProCA2-affi to the selected cells was first analyzed by immuno-fluorescence staining using the polyclonal antibody against PEGylated parental protein ProCA22. The cell binding analyses showed that the SKOV-3 cancer cells bind more ProCA22 compared to MDA-MB-231. These results indicated that the designed ProCA22 was able to target the cancer cells with high HER-2 expression level.

Cell binding analyses was applied to examine whether the designed ProCA22 can target to cancer cells. Binding of the Gd-ProCA22 to the selected cells was first analyzed by immuno-fluorescence staining using the polyclonal antibody against PEGylated parental protein. A substantial staining intensity of ProCA22 bound to SKOV-3 cells was observed. However, the MDA-MB-231 cells demonstrated very weak staining. The immunostaining results were consistent with NIR fluorescence imaging results. Under the assumption that $1 \times 10^7$ cells comprise a volume of 50 – 100 µL, this binding capacity led to the accumulation of Gd$^{3+}$ at 10 – 20 µM in the cell pellets. This local concentration is sufficient to produce strong MRI contrast, especially the protein contrast agent with high relaxivity reported here (Figure 7.9).
Figure 7.9. Cell targeting of CA22 for both cancer cells with high level (SKOV-3) and low level (mda-mb-231) of HER2 expression.

7.7. MR imaging and NIR images obtained from animals with the injection of designed protein based contrast

7.7.1. MR images obtained on 4.7 Tesla

MR images were acquired by applying the in vivo performance of MRI of CD-1 mice (20-25g, N = 4) on a 4.7 T Varian MRI system using a dedicated rodent coil with modified parameters and vary pulse sequences. During the MR scan, mice were anesthetized with 1.5% isoflurane and kept warm with a heated pad. MR images were acquired by T₁- and T₂-weighted fast spin echo sequences (TR=2 s, TE=0.022 s, and ESP = 0.01 s) with field of view of 3×3 cm, matrix of 256×256, and slice thickness of 1 mm. The tumors were dissected after MRI experiments. We examined the in vivo performance of MRI of CD-1 mice (20-25g, N = 4) using a 4.7 T Varian MRI system. Figures 7.10 and 7.11 shows the MR images of mice before and a series of time points after administration of approximately 100 µL of PEGylated Gd-ProCA22 (~3 mM) through the tail vein. Significant contrast enhancements were observed in several organs with the greatest enhancement in the kidney, liver, and blood vessels by comparison with pre- and post-contrast T₁ and T₂ weighted images obtained at 4.7 T. Meanwhile, the positive tumor injected SKOV-3 breast cancer cells with high HER-2 expression level gave stronger enhanced images compared with the negative tumors injected MDA-MB-231 with lower HER-2 expression level. The injection dose of 4.8 µmol kg⁻¹ was 20-fold lower than Gd-DTPA dose typically used in clinics (0.1 mmol/ kg). The tissue-dependent enhancement was consistent with the biodistribution of IHC staining results. We further observed, consistent with MR imaging, a strong NIR light emission from the SKOV-3 tumor at 24-hour post-administration of the contrast agent, however,
the NIR intensities at the MDA-MB-231 tumor site were much less than that of the SKOV-3 tumor (Figure 7.10 and 7.11).

Figure 7.10. MR images of mice with HER-2 tumor at 4.7 T with fast spin echo pulse sequence.

Special slices were selected to compare the images focusing on kidneys of this mouse. Images taken from post injected with ProCA22 at different time points (15 min, 3 hr, 26 hr and 51 hr) were compared to the pre-injection one.
Figure 7.11. MR images of xenograft tumor models in mice at 4.7 T using fast spin echo as the pulse sequence.

Slice 16 was selected to compare the images focusing on tumor of this mouse. Images taken from post injected with ProCA22 at different time points (15 min, 3 hr, 26 hr and 51 hr) were compared to the pre-injection one.
7.7.2. Near Infrared (NIR) imaging of ProCA22 labeled with Cy5.5

Cy5.5 Mono Maleimide dye produces an intense signal in the Near IR (NIR) region of the spectrum. NIR imaging has high sensitivity and is coupled with the MR imaging to confirm accurate targeting of selected biomarkers to cancer cells. We observed a strong NIR light emission from the SKOV-3 tumor at 24-hour post-administration of the contrast agent; however, the NIR intensities at the MDA-MB-231 tumor site were much less than that of the SKOV-3 tumor (Figure 7.12 and 7.13).

Here we introduced an optical imaging capability by conjugating a near-IR dye, Cy5.5 Mono Maleimide, to a Cystine residue at C-terminal of the protein to facilitate imaging analyses. The Cy5.5 Mono Maleimide has a maximum absorbance at 674 nm and a maximum emission wavelength at 689 nm. The labeling yield is as high as 70%; the unlabeled free dye was separated from labeled proteins by dialysis in 10 mM HEPES buffer. NIR images are shown before (Figure 7.12.A) and after (Figure 7.12.B) injecting ProCA22 into the mouse. It can be clearly observed that the kidney and liver were lit up 4 hours after injecting the contrast agent (Figure 7.12.C). Moreover, tumors and organs from the imaged mice were collected 48 hours post injection of ProCA22 to further analyze the HER2 targeting properties of the designed ProCA22 (Figure 7.13). The organs and tumors were imaged using optical animal imaging. High levels of accumulation of Cy5.5 in the liver, kidneys, and the SKOV-3 tumor were observed. In comparison, the level of Cy5.5 at the MDA-MB-231 tumor was quite low (Figure 7.12). The results strongly suggested that our protein contrast agent is able to target the tumor
with high HER-2 expression level and produce the HER-2 specific MR image enhancement.

Figure 7.12.NIR images of both positive tumor with SKOV-3 cell line and negative tumor with MDA-MB-231 cell line in the same mouse.

A, B). Image scanned before the administration of our designed contrast agent. C). Image scanned after the injection of ProCA22 for 4 hr.
Figure 7.13. Quantitative analysis of NIR images of tumor and organs from the mouse injecting ProCA22 after MR imaging.

A). NIR images of organs from the mouse with injection of ProCA22 post 48 hr.

B). Quantities analysis of NIR intensities from the images of organs on Figure A.
7.8. Immunohistion chemistry staining of tissues from the mice injected with our designed protein based contrast agent.

To further verify the contrast agent targeted to the HER-2 positive tumor, we applied immune histo-chemistry (IHC) staining using the antibody PAb with tissue slides made from the tumor samples and selected organs collected from the imaged mice. Strongest staining was observed with liver and the SKOV-3 tumor tissue slides (Figure 7.14). Close examination of the staining patterns of the tumor slides revealed distribution of the designed protein both inside and outside the cancer cells with substantial stronger staining inside the cancer cells, indicating internalization of the protein contrast agent. The kidney slides also presented strong immune staining consistent with the NIR imaging finding. Interestingly, the areas near proximal tubes showed strongest staining (Figure 7.14), suggesting that the protein contrast agent may be secreted through kidney. In contrast, immune staining of negative tumor sections grown from MDA-MB-231 cell line revealed very weak staining (Figure 7.14).
Figure 7.14. IHC staining of tissues from the mouse injected with ProCA22.
Figure 7.15. IHC staining of liver from the mouse injected with ProCA22.

A). over all image of IHC staining of liver. B). red color shows the protein stained by our antibody. C) Green color is the blood vessel stained by CD31. D). blue color is the nucleus acid.
7.9. Testing the toxicity of ProCA 22

The toxicities of the designed protein contrast agent were analyzed with CD-1 mice. No acute toxicity was observed upon the tail vein injection of 4-fold greater dosages than those used in MR Image over a 2-day test period. Characterization of serum samples from the tested mice receiving the agent suggested that no kidney, liver, and heart damages were detected (Figure 7.14).

![Graph of serum sample characterization](image)

**Normal Range:**
- **Na⁺:** 143-164 mmol/L
- **K⁺:** 6.3-8.0 mmol/L
- **Ca²⁺:** 4.6-9.6 mg/dL
- **Albumin:** 2.5-4.8 g/dL
- **Cholesterol:** 59-103 mg/dL
- **ALT:** 44-87 U/L
- **ALP:** 43-71 U/L

**Figure 7.16.** Toxicity test of the designed protein based MRI contrast agent with injection into the CD-1 mouse.

The salt (Na⁺, K⁺ and Ca²⁺) concentrations were measured and compared to the blank mouse which was injected with saline.
7.10. Gastrin Releasing Peptide and Gastrin Releasing Peptide Receptor

Gastrin-releasing peptide, also known as GRP, is an important regulatory molecule that has been implicated in a number of physiological and pathophysiological processes in humans. GRP is a bombesin structural related peptide with twenty-seven amino acids which was isolated from porcine gastric tissue recently. Gastrin-releasing peptide is a regulatory human peptide that elicits gastrin release and regulates gastric acid secretion and motor function. Bombesin is a 14-amino acid peptide originally isolated from the skin of a frog *Bombina bombina* in 1971. It has two known homologs in mammals called neuromedin B and gastrin releasing peptide. Bombesin and gastrin releasing peptide have the same amidated C-terminal heptapeptide. The gastrin-releasing peptide receptor (GRPR), also known as BB2 is a G protein-coupled receptor whose endogenous ligand is gastrin releasing peptide. GRPR mediates the function of GRP. GRP and its receptors are highly expressed in the pancreas in human.
Figure 7.17. Interrelationship between gastrin, histamine and somatostatin in control of gastric acid secretion\textsuperscript{178}.
Luminal acid directly inhibits the G-cells and gastrin release but stimulates the D-cells to release somatostatin which by paracrine pathway inhibits gastrin release from the G-cells and indirectly reduces gastric acid secretion. The H. pylori infection of antral mucosa increases gastrin release by acting on the G-cells through Nalpha-methylhistamine produced by infected mucosa and increase the release of proinflammatory cytokines that stimulate the G-cells to release gastrin and ECL-cells to release histamine, both leading to increased gastric acid secretion.

It has been reported that a high GRP expression level was detected in those neuroendocrine tumors, such as small cell lung cancer and androgen-dependent prostate cancer\textsuperscript{179}. GRP acts as growth factors to stimulate cell growth through its receptor (Gastrin Releasing Peptide Receptor) by autocrine and paracrine pathways\textsuperscript{180}. The expression level of GRPR has been found to correlate with tumor stages. The binding affinity of GRPR to its agonist is variable and depends on the type of cells and agonists. GRPR was found to be aberrantly expressed by various cancer cells, which suggests potentially important clinical impacts for future drug development. Based on the importance of GRP, we are going to apply our protein-based contrast agents to include specific targeting to the GRPR and detecting the prostate cancer through MR imaging.
8. Developing Protein Based Contrast Agents using Half Domains of CaM

8.1. Introduction

The properties of Ca$^{2+}$ binding to CaM have been well studied since CaM is the most important Ca$^{2+}$ binding regulatory protein. However, the impact of Mg$^{2+}$ on Ca$^{2+}$ binding to CaM is important physiologically, and has long been a matter of debate. Baudier, J. et al had proposed that not only Ca$^{2+}$ but also Zn$^{2+}$ can bind to the S100 proteins and result in conformational changes. They also pointed out the binding sites for Ca$^{2+}$ and Zn$^{2+}$ are quite distinct. The tryptic fragments of CaM have been shown to be very similar to the corresponding domains of intact CaM in terms of 3D structures as well as Ca$^{2+}$ binding characteristics. Shea MA et al have reported inter domain cooperativity of paramecium calmodulin bound to melittin preferentially increases calcium affinity of sites I and II. We have discussed the Ca$^{2+}$ binding properties of these half domain CaM subunit in Chapter 6, and we are going to continue analyze the binding affinities of Zn$^{2+}$ and Lanthanides in this chapter.

Signal intensity in MRI was produced mainly from the local value of the longitudinal relaxation rate $1/T_1$ and the transverse rate, $1/T_2$ of water protons. Signal is expected to increase with shortening $T_1$ and decrease with longer $T_2$. Contrast agents increase both $1/T_1$ and $1/T_2$ rates to varying degrees depending on their nature as well as the applied magnetic field strength. Contrast agents such as gadolinium (III) are selected for $T_1$-weighted images since the percentage change in $1/T_1$ in tissue is much greater than that in $1/T_2$ because they can increase $1/T_1$ and $1/T_2$ at similar amounts. The
presence of a gadolinium (III) complex will increase the longitudinal and transverse relaxation rates, $1/T_1$ and $1/T_2$, respectively, of solvent nuclei$^{184}$. Diamagnetic and paramagnetic relaxation rates are additive and given by \textbf{Equation 8.1}, where $(1/T_i)_{\text{obs}}$ is the observed solvent relaxation rate and the subscripts “d” and “p” refer to diamagnetic and paramagnetic, respectively. The paramagnetic contribution is dependent on the concentration of paramagnetic species. Relaxivity, $r_i$, is defined as the slope of the concentration dependence as observed in \textbf{Equation8.2}. Thus a plot of $(1/T_i)_{\text{obs}}$ versus concentration would give the relaxivity as the slope. Relaxivity is normally expressed in units of mM$^{-1}$ s$^{-1}$; however, molal concentrations should be used when dealing with non-dilute systems$^{185}$. The origin of paramagnetic relaxation enhancement is generally divided into two components, inner sphere and outer-sphere as shown in \textbf{Equation8.3}. Inner-sphere relaxation refers to relaxation enhancement of a solvent molecule directly coordinated to the paramagnetic ion, and outer-sphere relaxation refers to relaxation enhancement of solvent molecules in the second coordination sphere and beyond (i.e., bulk solvent). This separation is used in an attempt to explain observed relaxivities in terms of existing theories$^{186}$. As in the previous review, the emphasis will be on the longitudinal relaxation rate $(1/T_1)$ enhancement of water hydrogen atoms since this is the effect which is of most interest in MRI$^{139a}$.

$$\frac{1}{T_i}_{\text{obs}} = \frac{1}{T_i}_d + \frac{1}{T_i}_p \quad i = 1, 2 \quad \text{Equation8.1}$$

$$\frac{1}{T_i}_{\text{obs}} = \frac{1}{T_i}_d + r_i[Gd] \quad i = 1, 2 \quad \text{Equation8.2}$$

$$\frac{1}{T_i}_p = \frac{1}{T_i}_{\text{inner-sphere}} + \frac{1}{T_i}_{\text{outer-sphere}} \quad i = 1, 2 \quad \text{Equation8.3}$$

As discussed in \textbf{Chapter 7}, MRI provides good contrast between the different soft tissues of the body, which make it especially useful in imaging the brain, muscles, the
heart, and different cancers. Moreover, MRI scans are fast and harmless to the patient\textsuperscript{184, 187}. Utilizing strong magnetic fields and non-ionizing radiation in the radio frequency range unlike CT scans and traditional X-rays, which both use ionizing radiation\textsuperscript{188, 189}. We have already created a protein based contrast agent, ProCA22, with a HER-2 affibody fused into the compact CaM. Enhanced images were obtained with both 4.7 and 7 tesla Varian MRI instruments with lower dosage injections of ProCA22. However, more space exists for further improvement of this contrast agent. For example, it took more than 2 hours to target to the tumor and most contrast agents accumulated in the liver and kidney instead of the tumor.

In this chapter, we report our studies to optimize the design of MRI contrast agents by using half domain of CaM, which not only have smaller molecular weight, but also abolish most of their biological function in cells. We first examined metal binding for Zinc and Gd. We then examine their relaxarion properties using 1.4 T relaxometer.

8.2. Metal binding affinities and selectivity of wild type CaM and half domains determined by fluorescence spectroscopy

Gd\textsuperscript{3+} and Zn\textsuperscript{2+} binding affinities of wild type and the mutant CaMs were determined by competition fluorescence titration methods using Fura-6F and Fluozin-1 (metal ion indicators, Invitrogen Molecular Probes) as Gd\textsuperscript{3+} and Zn\textsuperscript{2+} indicator respectively. The fluorescence spectra of Fura-6F and Fluozin-1 were acquired using a fluorescence spectrophotometer (Photon Technology International, Inc.) with a 10 mm path length quartz cell at 25 °C. Fura-6F excitation spectra were acquired at two wavelengths, 340
and 380 nm, with an emission wavelength at 510 nm. Fluozin-1 emission spectra were acquired at 495 nm with emission wavelength from 500 to 600 nm. Gd\(^{3+}\)-binding affinity of Fura-6F, Zn\(^{2+}\) binding affinity of Fluozin-1, the \(K_{d1}\), were first determined by a Gd\(^{3+}\)/Zn\(^{2+}\) titration to the dyes. A solution of Fura-6F/Fluozin-1 was mixed with Gd\(^{3+}\)/Zn\(^{2+}\) at a 1:1 ratio for a competition titration accompanied with gradual addition of proper amount of protein aliquots. An apparent dissociation constant, \(K_{app}\), was estimated by fitting the fluorescence excitation intensity ratio of Fura-6F at 340 and 380 nm and Fluozin-1 at 520 nm with a various concentrations of wt CaM and its mutants by a 1 to 1 binding model. The real Gd\(^{3+}\)/Zn\(^{2+}\) binding affinity to CaMs, the \(K_{d2}\), were calculated with the following equation:

\[
K_{d2} = K_{app} \times \frac{K_{d1}}{K_{d1} + [dye]_r} \quad \text{Equation 8.4}
\]

Zinc binding affinities to these mutants were determined by dye competition method by monitoring the fluorescence signals (Figure 8.1). CaM displays higher binding affinities to Lanthanide metal ions, such as Gd\(^{3+}\) (Figure 8.4) and Tb\(^{3+}\) (Figure 8.3) with the \(K_d\) about \(\sim 10^{-12}\) M range. From the summary in Table 8.1, the two domains of CaM have varied affinities to Tb\(^{3+}\) and Gd\(^{3+}\) with 4 folds higher affinity for Gd\(^{3+}\) in the N–terminal domain (0.5 \(\times 10^{-12}\) M) than C–terminal domain (2.0 \(\times 10^{-12}\) M). However, the \(K_d\) of C–terminal CaM is \(\sim 7.9 \times 10^{-12}\) M, which is similar as wild type (7.0 \(\times 10^{-12}\) M), but the N–terminal CaM cannot be obtained due to the limitations associated with method requirement. In addition, Gd\(^{3+}\) has a smaller \(K_d\) than Tb\(^{3+}\) for both domains of CaM. CaM with the five residues deleted in the linker range has almost two folds stronger binding.
affinities for Tb\(^{3+}\) (~4.5 \times 10^{-12} \text{ M}) and Gd\(^{3+}\) (~3.7 \times 10^{-12} \text{ M}) compared to wild type (~7.0\times10^{-12} \text{ M for Tb}^{3+} \text{ and } 5.0 \times 10^{-12} \text{ M for Gd}^{3+}).

Figure 8.1. Zn\(^{2+}\) binding affinity to Fluozin1 by fluorescence titration.
Figure 8.2. Zn$^{2+}$ binding affinity determined using protein competing with the fluozin-1. Data were fitted by 1 to 1 equation.

Figure 8.3. Gd$^{3+}$ binding affinity determined using protein competing with the Fura-6F. Data were fitted by 1 to 1 equation.
Figure 8.4. Tb$^{3+}$ binding affinity determined FRET. Data were fitted by hill equation.

Table 8.1. Summary of metal binding affinities of wild type CaM and the half domains.

<table>
<thead>
<tr>
<th>$K_d$ (x10^{-12}), M</th>
<th>wt-CaM</th>
<th>N-CaM</th>
<th>C-CaM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Ca^{2+}$</td>
<td>$6.77 \times 10^6 \pm 0.4$</td>
<td>$6.43 \times 10^6 \pm 0.1$</td>
<td>$1.18 \times 10^6 \pm 0.2$</td>
</tr>
<tr>
<td>$Zn^{2+}$</td>
<td>$1.82 \times 10^6 \pm 0.2$</td>
<td>$1.11 \times 10^6 \pm 0.1$</td>
<td>$0.0027 \times 10^6 \pm 0.3$</td>
</tr>
<tr>
<td>$Tb^{3+}$</td>
<td>$7.0 \pm 0.1$</td>
<td>N/A</td>
<td>$7.9 \pm 0.2$</td>
</tr>
<tr>
<td>$Gd^{3+}$</td>
<td>$0.50 \pm 0.1$</td>
<td>$0.50 \pm 0.1$</td>
<td>$2.0 \pm 0.1$</td>
</tr>
</tbody>
</table>
Lobe-specific Ca\(^{2+}\) binding affinities of the CaM variants were determined via monitoring intrinsic fluorescence changes during equilibrium calcium titrations as previously described\(^{57,58}\). CaM / Ca\(^{2+}\) dissociation constants (\(K_d\)) and Hill coefficients (\(n_H\)) as well as free energy were derived from these fits and summarized in Chapter 3. As previously established, C-terminal lobe Ca\(^{2+}\) binding occurred with a significantly higher affinity that N-terminal Ca\(^{2+}\) binding in the compact CaM protein\(^{102}\); the isolated C – terminal domain still exhibits more than 5- folds stronger affinity than isolated N – terminal domain. While the Ca\(^{2+}\) binding affinity to both the isolated N – terminal domain and C –terminal domain increased 2-fold compared to the compact protein. These results confirmed the cooperative Ca\(^{2+}\) binding properties between the two domains of CaM. The Hill numbers for both the two domains are close to one instead of two in the wild type CaM.

CaM and its variants exhibited much stronger binding affinities to lanthanide metal ions (Tb\(^{3+}\) and Gd\(^{3+}\)) than Ca\(^{2+}\). The log pKa of CaM and its variants to Tb\(^{3+}\) and Gd\(^{3+}\) are 6 times greater than those to Ca\(^{2+}\). Based on the fact that oxygen is the only chelator in the metal binding pocket of CaM, and that lanthanide metal ions have higher interaction ability to oxygen, the binding affinities we obtained are reliable. As previously established, Ca\(^{2+}\) has higher binding affinities in the C –terminal domain of CaM than N – terminal domain. However, the lanthanide metal ions show an opposite trend where the N – terminal domain has stronger binding affinities than C- terminal domain. The \(K_d\) of Gd\(^{3+}\) is slightly lower than Tb\(^{3+}\) indicating that it has higher binding affinity than Tb\(^{3+}\).
8.3. Relaxivities of CaM half domains detected on the 60 MHz.

Relaxivity is used to improve the contrast of the image, and to study tissue specific areas where the contrast agent better diffuses or to perform functional magnetic resonance imaging\(^{135b}\). It refers also to the ability of magnetic compounds to increase the relaxation rates of the surrounding water proton spins. The relaxivity of MRI contrast agents depends on the molecular structure and kinetics of the complex. To increase the number of water molecules in the inner sphere of the complex, or to slow down the molecular rotational correlation time, are possibilities to improve the water relaxivity.

For CaM, we applied the 1.41 T relaxometer to measure both the \(r_1\) and \(r_2\) relaxivities of these CaM variants and further study the properties of the engineered N- and C- domain, and the deletion linker region of CaM (Fig 8.6.A and B) as well as the modified CaM variants by PEGylation with PEG40 (Fig 8.6.C and D). Purified protein samples were prepared in 10 mM HEPES buffer, pH 7.4 at concentrations of 5, 10, 20, and 40 µM mixed with Gd\(^{3+}\) as 1 to 2 ratio for half domains and 1 to 4 ratio for wt CaM and Del-CaM. The experiments were completed at 37 °C maintained by the water bath.
Figure 8.5. Concentration dependence of relaxivities of CaM and the isolated variants bond with Gd$^{3+}$ as 1 to 4 ratios on 1.41 T.

$r_1$ and $r_2$ relaxivities calculated from $T_1$ and $T_2$ relaxation times measured on 1.41 T of isolated N-(black circles), C- domain (blue squares) and CaM with the linker region deleted (green diamond). A). $r_1$ relaxivities of CaM variants. B). $r_2$ relaxivities of CaM variants. C). $r_1$ relaxivities of Pegylated CaM variants. D). $r_2$ relaxivities of Pegylated CaM variants.
Table 8.2 shows the relaxivities of isolated half domains and Del-CaM as well as the modified mutants by PEGylation. All of these mutants of CaM prohibited high relaxivities especially the $r_2$ relaxivity. Del –CaM showed the highest relaxivities compared to others. In addition, modification of CaM half domains by PEGylation with (PEG)$_{40}$ does not affect their relaxivities apparently. This may be due to the fact that many of the lysine residues used for PEGylation were not in the binding site and the relaxivities were not changed after PEGylation$^{135b,190}$.

Table 8.2. Relaxivities of wt-CaM, CaM variants and PEGylated variants.

<table>
<thead>
<tr>
<th></th>
<th>wt-CaM</th>
<th>Del-CaM</th>
<th>N-CaM</th>
<th>N-CaM-p40</th>
<th>C-CaM</th>
<th>C-CaM-p40</th>
</tr>
</thead>
<tbody>
<tr>
<td>$r_1$(mM$^{-1}$s$^{-1}$)</td>
<td>47.9</td>
<td>75.7</td>
<td>49.3</td>
<td>46.0</td>
<td>30.7</td>
<td>33.2</td>
</tr>
<tr>
<td>$r_2$(mM$^{-1}$s$^{-1}$)</td>
<td>53.3</td>
<td>106.2</td>
<td>63.7</td>
<td>62.5</td>
<td>41.1</td>
<td>44.6</td>
</tr>
</tbody>
</table>
9. PEGylation of Protein Based MRI Contrast Agents Improves MRI Properties

9.1. Introduction

We have reported the development of protein-based MRI contrast agents (ProCA1, previously named as CA1.CD2) by rational design of Gd$^{3+}$ binding sites into a stable protein using amino acid residues as metal coordinating ligands. Our designed specialized Gd-binding protein-based contrast agents are different from other reported macromolecular MRI contrast agents, for example, Gd-DTPA-based dendrimers, or nano-sized contrast agents that are developed by multiple attachment of an existing small chelator for Gd$^{3+}$. Our designed protein MRI contrast agent exhibits higher $r_1$ and $r_2$ relaxivities compared to Gd-DTPA. Here, we report a further improvement in the design of protein based contrast agents ProCA1 for in vivo imaging by protein modification with various sizes of polyethylene glycol (PEG) chain (‘PEGylation’). Our experimental results demonstrate that modified contrast agents exhibit significant improvement of in vivo MR imaging and biocompatibilities including increase in relaxivities, protein solubility, blood retention time and decreased immunogenicity.
9.2. Results and discussion

9.2.1. Protein engineering and PEGylation

Bacteria expressed and purified protein ProCA1 was PEGylated with Methyl-PEO$_n$-NHS esters with different molecular weights of 0.3, 0.6, 2.4, 5, 12 and 20 kDa, respectively, and further purified by FPLC and confirmed by the MALDI-TOF mass spectrometric analysis with one to three PEG chains attached. First, we confirmed that PEGylated proteins maintain a native structure monitored by Trp fluorescence. PEGylated ProCA1 exhibited similar disassociation constants for Gd$^{3+}$ with a 1 to 1 binding ratio to those of ProCA1 (Figure 9.1 and Table 9.1).
The Gd$^{3+}$-binding affinity of native ProCA1 was determined by a competition titration using commercially available fluorophore Fluo-5N as a Gd$^{3+}$ indicator.

Table 9.1. Properties of Gd-DTPA, ProCA1 and its PEGylated variants.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Gd-DTPA</th>
<th>ProCA1 0.6k</th>
<th>ProCA1 2.4k</th>
<th>ProCA1 12k</th>
</tr>
</thead>
<tbody>
<tr>
<td>D ($\times 10^7$ cm$^2$s$^{-1}$)</td>
<td>-</td>
<td>13.9</td>
<td>12.9</td>
<td>12.1</td>
</tr>
<tr>
<td>Radius (Å)</td>
<td>-</td>
<td>16.9</td>
<td>18.2</td>
<td>19.4</td>
</tr>
<tr>
<td>Log $K_a$</td>
<td>22.45*</td>
<td>11.46</td>
<td>11.26</td>
<td>11.70</td>
</tr>
<tr>
<td>q</td>
<td>1.1#</td>
<td>2.4</td>
<td>3.2</td>
<td>3.0</td>
</tr>
<tr>
<td>$r_1$ (mM$^{-1}$s$^{-1}$)</td>
<td>4.5</td>
<td>22.9</td>
<td>34.4</td>
<td>55.6</td>
</tr>
<tr>
<td>$r_2$ (mM$^{-1}$s$^{-1}$)</td>
<td>5.2</td>
<td>28.7</td>
<td>40.8</td>
<td>65.6</td>
</tr>
</tbody>
</table>

* From reference 11. # From reference 12.

Table 9.1 shows that the native ProCA1 had a hydration number of 2.4, PEGylation resulted in an increase of hydration number 0.2 - 0.8. Subsequently, the hydrodynamic radii of modified ProCA1s were determined by a pulse-field-gradient diffusion NMR and it increases from 16.9 ± 2.0, to 18.2 ± 1.1, 19.4 ± 0.55 and 40.2 ± 0.2 Å, with 0.6, 2.4 and 12 kDa PEG respectively (Table 9.1 and Figure9.4).
9.2.2. Relaxivity and metal binding affinity measurement

Next, we observed that the PEGylation modifications dramatically increased longitudinal and transverse relaxivities of the ProCA1 at different field strengths tested (0.47, 1.4, 3.0 and 9.4 T). The $r_1$ and $r_2$ values of ProCA1-PEG0.6k were increased almost 66 and 110%, and ProCA1-PEG2.4k increased 100 and 125%, respectively. The ProCA1-PEG12k exhibited 19-fold higher $r_1$ and $r_2$ values compared with Gd-DTPA (Figure 9.2 and Table 9.1). More interestingly, at high field strength of 9.4 T, ProCA1-PEG2.4k still exhibited great increase of relaxivities for $r_1$ and $r_2$. The number of water ligands coordinated to the Gd$^{3+}$ ProCA1 complex was determined by measuring Tb$^{3+}$ luminescence decay in H$_2$O or D$_2$O. The Tb$^{3+}$ excited-state lifetime was measured using a fluorescence spectrophotometer (Photon Technology International, Inc.) with a 10 mm path length quartz cell at 22 °C. Following excitation at 265 nm with a XenoFlash lamp (Photon Technology International, Inc.), Tb$^{3+}$ emission was monitored at 545 nm in a time series experiment in both H$_2$O and D$_2$O systems. Luminescence decay lifetime was obtained by fitting the acquired data with a mono exponential decay function. H$_2$O in ProCA1 solution was replaced with D$_2$O by lyophilization and re-dissolved in D$_2$O for at least three times.
Figure 9.2. Relaxivity values on vary magnetic field strengths.

Relaxivity values $r_1$ (A) and $r_2$ (B) at 3.0 (blue) and 9.4 T (red) of Gd-DTPA, ProCA1 and its PEGylated variants. Relaxivity values $r_1$ (C) and $r_2$ (D) of Gd-DTPA (blue), ProCA1 (red), ProCA1-PEG0.6k (yellow), and ProCA1-PEG2.4k (green) at different field strengths.
Figure 9.3. Measurement of Water Coordination Number of protein based contrast agents by Tb$^{3+}$ lifetime.
Figure 9.4. Hydrodynamic radii of ProCA1 measured on 600 MHz.

(*) and PEGylated with variant PEGs, PEG0.6k (●), PEG2.4k (■) and PEG12k (▲), were determined by pulse-field-gradient diffusion on 600 MHz NMR. PEGylation increased the radii gradually based on PEG size increased. Data were fitted by one component equation: \( \exp (-m1^*m0^2) \).
9.2.3. **In vivo toxicity test**

The ProCA1 variants also exhibit low *in vivo* toxicity. Metal complexation is a key mediator or modifier of enzyme structure and function, especially group IA metals Na\(^+\) and K\(^+\) play important and specific roles that assist function of biological macromolecules. Ca\(^{2+}\) plays a pivotal role in the physiology and biochemistry of organisms and the cell. As a result, it is essential to test the chemical properties. Selected metal ion concentrations (K\(^+\), Na\(^+\) and Ca\(^{2+}\)) and enzyme activity were measured from the collected blood of mice with the injection of ProCA1 and the PEG variants (Research Animal Diagnostic Laboratory, University of Missouri). The results (*Table 9.2*) show that all the metal ion concentrations of the mice injected with ProCA1-PEGs are in the same level as those with saline. In addition, alanine transaminase, the enzymes related with liver function, is in the normal level for mice with administration of ProCA1-PEGs compared to those injected with saline. However, another enzyme, alkaline phosphatase (ALP), which indicates the function of kidney; was tested to have fewer amounts for the mice injected with ProCA1-PEGs than those with saline. In summary, we were able to say that no apparent *in vivo* toxicity of ProCA1 is observed compared with the control group.
Table 9.2. *In Vivo* Toxicity of designed protein contrast agents.

<table>
<thead>
<tr>
<th></th>
<th>Sodium</th>
<th>Potassium</th>
<th>Calcium</th>
<th>Creatinine</th>
<th>ALT</th>
<th>ALP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mmol/L)</td>
<td>(mmol/L)</td>
<td>(mg/dL)</td>
<td>(mg/dL)</td>
<td>(U/L)</td>
<td>(U/L)</td>
</tr>
<tr>
<td>ProCA1</td>
<td>151.9±1.6</td>
<td>11.7±0.5</td>
<td>11.2±0.1</td>
<td>0.27±0.02</td>
<td>32±4</td>
<td>22±1</td>
</tr>
<tr>
<td>ProCA1-PEG0.6k</td>
<td>150.8±4.0</td>
<td>10.3±1.4</td>
<td>10.5±0.5</td>
<td>0.22±0.02</td>
<td>49±4</td>
<td>35±4</td>
</tr>
<tr>
<td>ProCA1-PEG2.4k</td>
<td>156.3±2.0</td>
<td>11.2±0.5</td>
<td>10.2±0.2</td>
<td>0.20±0.02</td>
<td>65±8</td>
<td>38±6</td>
</tr>
<tr>
<td>Saline</td>
<td>151.8±11.0</td>
<td>11.8±2.3</td>
<td>11.0±0.5</td>
<td>0.32±0.01</td>
<td>39±5</td>
<td>89±11</td>
</tr>
</tbody>
</table>

9.2.4. *In vivo* Imaging and specifically cell targeting

PEGylation increased the solubility of protein contrast agents in aqueous solutions 20- to 100-fold, most likely due to the strong hydrophilicity of the PEG chains. Significant increases in the solubility of contrast agents permit us to perform *in vivo* studies. **Figure 9.5A** shows the $T_1$-weighted fast spin-echo MR images of the mouse before (left) and 0.5 hr after administration (right) of PEGylated Gd-ProCA1 (dose of 4.8 μmol kg$^{-1}$) through tail vein injection. Significant contrast enhancements were observed in the kidney, liver, and blood vessels by comparison with pre- and post-contrast $T_1$ weighted images. The injection dose used was 20-fold lower than Gd-DTPA dose typically used in clinics (0.1 mmol kg$^{-1}$). The tissue dependent enhancement was consistent with the biodistribution of $^{153}$Gd isotope analysis.
(A) T1-weighted fast spin-echo (TR = 2 s, TE = 0.022 s, ESP = 0.01 s, slice thickness = 1 mm) MR images of a mouse before (left) and 0.5 hr after administration (right) of Gd-ProCA1-PEG2.4k. (B) ELISA of antibody produced in rabbits serum before (Pre) and weeks 3 (black) and 6 (red) after i.p. injection of ProCA1 and ProCA1-PEG2.4k mixed with adjuvant (ProCA1-Adjuvant) or with buffer saline (ProCA1). PabCD2 is the anti-serum from rabbits produced by CD2 (commercial source) as antigen. (C) Two types of cancer cell lines were stained by ProCA1-affi and ProCA1-affi-PEG2.4k respectively. SKOV-3 is overexpressed with HER2 receptor. MDA-MB-231 is negative of HER2. Red: ProCA1-affi or ProCA1-affi-PEG2.4k; Blue: DAPI for nucleolus.

Figure 9.5. MRI and cell targeting study of ProCA1.
9.2.5. Blood retention and immunogenicity

The effect of PEGylation on blood retention and biodistribution of ProCA1 and its PEGylated variants in mice were then examined by immunochemical assays (Figure 9.6A) and γ-radiation of $^{153}$Gd isotope (Figure 9.6C). PEGylation increased the blood retention time of the agents. ProCA1-PEG2.4k exhibited the greatest increase in blood retention time. PEGylation reduced immunogenicity. Rabbits were administrated intraperitoneally with Gd-ProCA1 and its PEGylated variants mixed with or without adjuvant according to the standard protocol. The rabbits were subjected to repeat immunizations over a 3-week interval. Blood samples were examined by ELISA (Figure 9.5B) and immunoblotting (Figure 9.7) using polyclonal antibody PabCD2 as positive control and the pre-bleed as negative controls. Our results suggest that the immunogenecity of the protein contrast agent may not be very strong, especially without addition of adjuvant. PEGylation substantially reduced the immune responses monitored by poly-antibodies. Antibody generativity against the protein contrast agent (both PEGylated and native protein) in the rabbits was examined by Western blotting. The experiments were carried out by SDS-PAGE of ProCA1-PEG2.4k, ProCA1-PEG0.6k and ProCA1 and detected by anti-serum collected from immuno-inoculated rabbits.

Appropriate dose of Gd-ProCA1-PEG2.4k, Gd-ProCA1-PEG0.6k and Gd-ProCA1 were injected intravenously into the mouse via the tail vein. Blood samples (~50 μL) were collected via orbital sinus at different time points. The contents of the protein MRI contrast agents in the blood were monitored by dot-blotting. Equal volumes of serum were loaded on the plate and identified by the commercial CD2 polyclonal antibody PabCD2. The amount of the ProCA1-PEG2.4k (1) and ProCA1-PEG0.6k (2) left in blood serum were higher than ProCA1 (3) at 3 hr. At 19 hrs, the amount of
ProCA1-PEG2.4k (4) left in blood serum is more than ProCA1-PEG0.6k (5) and ProCA1 (6). (B) Is control with different amount of ProCA1. The results show that the longer blood retention time was observed for the protein with higher degree of PEGylation and longer PEG chain modifications. Although the addition of adjuvant indeed resulted in a strong immunoresponse it was clear that without adjuvant, injection of ProCA1 did not lead to significant antibody production even after a double dose immunization suggesting that the immunogenicity of the ProCA1 is not strong, especially without addition of adjuvant. Additionally, this immunoresponse was dramatically reduced (~80%) by PEGylation with PEG2.4k. This data is consistent with observation for other protein drugs¹⁹¹.
Figure 9.6. PEGylation increased *in vivo* blood retention time of the agent in the mouse model.

A). Dot Blotting and B) γ-radiation of $^{153}$Gd isotope. (C) Accumulation of $^{153}$Gd in blood circulation (% of total injected dose) for GdCl$_3$, ProCA1, ProCA1-PEG2.4k, and ProCA1-PEG12k at time points of 1 (blue), 4 (brown), 8 (yellow) and 12 hrs (green) after injection.
We have also created a targeted contrast agent against the HER-2 receptor over expressed in various cancer cells by fusion of a HER-2 affibody \(^{192}\) to the C-terminal of ProCA1 (denoted as ProCA1-affi). Fig. 9.5C shows that both ProCA1-affi and ProCA1-affi-PEG2.4k have similar capability to specifically bind to SKOV-3 ovarian cancer cells with HER2 highly expressed (~3 \(\times\) 10^6/cell). On the other hand, they do not bind to MDA-MB-231 cancer cells with a low expression level of HER-2 (~2.7 \(\times\) 10^3/cell) \(^{192}\). Therefore, PEGylation does not impede targeting capability and specificity of ProCA1 reagents, which is important for molecular imaging of biomarkers.

Figure 9.7: The other antiserum was prepared from the blood collected after two times of injection of native protein ProCA1 mixed with adjuvant.

(A). ProCA1 in the absence of adjuvant (B). ProCA1-PEG2.4k mixed in absence of adjuvant (C). The results showed that addition of adjuvant induced stronger immune responses (B). PEGylation modifications of the protein dramatically reduced immune responses in the rabbits (C). The commercial CD2 polyclonal antibody PabCD2 was used as positive control (D).
9.3. Conclusions

Different from the modest effect (15% increase) reported \textsuperscript{139a} for a small chelator as contrast agent, here we reported the first observation of significant relaxivities increase of protein contrast agents (up to 200%) by PEGylation. Understanding the mechanisms for the increased relaxivity by PEGylation may allow us to identify new key factors contributing to the substantial improvement in relaxivities which is the major challenge in the field of molecular and biomarker targeted imaging. PEGylation may be a novel avenue to increase relaxivity at high field strength to overcome the limitation of current small Gd\textsuperscript{3+} chelate contrast agents that suffer reduction of $r_1$ relaxivity under a high magnetic field (Figure 9.2). Such improvement can be important to the animal imaging and pre-clinical research under high or ultra-high field where there is an urgent need for molecular imaging probes and optimized contrast agent.
10. Significance of This Study

10.1. Review

CaM is a small (148 amino acids; MW: 16.7 KDa) intra cellular protein with pI ~4.0. It consists of two globular and autonomous domains, each of which contains two helix-loop-helix EF-hand motifs. CaM is featured as a Ca$^{2+}$ binding protein with the cooperatively binding properties between its two domains. Through its reversible or irreversible binding to Ca$^{2+}$, the flexible conformational changes and the interactions with target enzymes, CaM is capable to transduce the intracellular Ca$^{2+}$ signal changes into a number of diverse cellular events, including apoptosis, cell differentiation and cell proliferation. Ca$^{2+}$ is one of the most important second messengers in eukaryotic cells. Temporal and spatial changes of the Ca$^{2+}$ concentration in different compartments of cells affect the regulation of cellular signalling. Among myriad of Ca$^{2+}$-binding proteins which modulate cell signalling, CaM plays an essential role and is extensively studied due to its ubiquitous expression in eukaryotes in addition to its versatile ability to activate or inhibit more than 100 functional enzymes, cellular receptors and ion channels. Although quite a few studies have been done on CaM, there are still large numbers of unknown scopes for this protein. For example, the binding sites of CaM to distinct enzymes are different and it may relate with the function. This study aims to investigate the structural and functional characteristics of CaM. We attempt to answer the following questions: 1. What is the metal selectivity and binding affinity of CaM except to Ca$^{2+}$? 2. What are the contributions from the structural components of CaM on regulating the biological functions? 3. Can we really tune the biological functions of those targeted
enzymes by varying the Ca\textsuperscript{2+} binding affinities to CaM? 4. How to determine the binding modes of CaM with targeted proteins and what are the differences between Ca\textsuperscript{2+}-CaM and Apo –CaM binding? 5. Can we engineer CaM to be applied as a MRI contrast agent?

10.2. The selectivity of metal binding and the contributions from the structural components of CaM on regulating the biological functions.

The two domains of CaM exhibit diversities as well as similarities. They have similar secondary structures while different tertiary structures. They produce close relaxivity value with varying metal binding affinities. Chapter 6 reports the metal selectivities and binding affinities of CaM with Ca\textsuperscript{2+}, Zn\textsuperscript{2+}, Tb\textsuperscript{3+} and Gd\textsuperscript{3+} for the isolated half domains. The Del-CaM and isolated half domain metal affinities were measured using the same method as wt CaM. The half domains of CaM exhibited stronger binding affinities to Ca\textsuperscript{2+} than they have in the compact wild type CaM, which indicates that Ca\textsuperscript{2+} undergoes cooperative binding to the two domains in CaM. Tb\textsuperscript{3+} and Gd\textsuperscript{3+} have $K_d$ values 10\textsuperscript{6} times smaller than Ca\textsuperscript{2+} and Zn\textsuperscript{2+} indicating that lanthanides bind to CaM with stronger affinities. This is an advantage for CaM as a MRI contrast agent (Chapter 7 and 8). The melting temperature measured by CD spectra suggested that all these structural components decreased their stability compared with wild type CaM (twice decrease in the $T_m$ values). In Chapter 3, PDE function assay using time based fluorescence spectroscopy was selected as an example to study the functional roles of structural component CaMs. The activity of CaM on PDE was eliminated after deleting five residues in the central linker and the activity was greatly decreased in the isolated C-terminal domain. However, the N-terminus was able to maintain its activity with a similar
EC\textsubscript{50} as the wild type CaM. These results demonstrate that the N – terminal domain of CaM is the key factor to the activity on PDE activity. While C – terminal and the central linker region may contribute less to the interactions between CaM and PDE.

10.3. **Tune the biological functions of those targeted enzymes by varying the Ca\textsuperscript{2+} binding affinities to CaM.**

A number of skeletal muscle diseases including malignant hyperthermia (MH) and Duchenne muscular dystrophy (DMD) are genetic disorders leading to impaired cellular Ca\textsuperscript{2+} regulations. Mutations in RyR1 account for the majority of MH cases \textsuperscript{89} and leaky RyR1 channel may contribute to DMD \textsuperscript{90}. RyR1 channels from MH susceptible individuals exhibit an enhanced sensitivity to CaM activation \textsuperscript{91,92} and possibly a decreased sensitivity to Ca\textsuperscript{2+}-CaM inhibition \textsuperscript{93}. CaM shifts the biphasic Ca\textsuperscript{2+} dependence of RyR1 activation leftward, effectively increasing channel opening at low Ca\textsuperscript{2+} and decreasing channel opening at high Ca\textsuperscript{2+}. The conversion of CaM from a RyR1 activator to an inhibitor is due to the binding of Ca\textsuperscript{2+} to CaM, however which of CaM’s four Ca\textsuperscript{2+} binding sites serves as the switch for this conversion is unclear. In Chapter 4, we engineered a series of mutant CaMs designed to individually increase the Ca\textsuperscript{2+} affinity of each of CaM’s EF-hands by increasing the number of acidic residues in Ca\textsuperscript{2+} chelating positions. Domain-specific Ca\textsuperscript{2+} affinities of each CaM variant were determined by equilibrium fluorescence titration. Mutations in sites I (Thr26Asp) or II (Asn60Asp) in CaM’s N-terminal domain had little effect on CaM Ca\textsuperscript{2+} affinity and regulation of RyR1. However, the site III mutation Asn97Asp increased the Ca\textsuperscript{2+} binding affinity of CaM’s C-terminal domain and caused CaM to inhibit RyR1 at a lower Ca\textsuperscript{2+} concentration than wild type CaM. Conversely, the site IV mutation Gln135Asp decreased the Ca\textsuperscript{2+} binding
affinity of CaM’s C-terminal domain and caused CaM to inhibit RyR1 at higher Ca\(^{2+}\) concentrations. These results support the hypothesis that Ca\(^{2+}\) binding to CaM’s C-terminal acts as the switch converting CaM from a RyR1 activator to a channel inhibitor. These results further indicate that targeting CaM’s Ca\(^{2+}\) affinity may be a valid strategy to tune the activation profile of CaM-regulated ion channels. Therefore, targeting these muscles with a CaM that will reduce channel opening at low Ca\(^{2+}\) may be of therapeutic benefit. Further, the enhanced Ca\(^{2+}\)-CaM inhibition of RyR1 may reduce the resting Ca\(^{2+}\) leak through the channels in DMD muscle.

10.4. **Determine the binding mode between CaM and target enzymes.**

As we know, CaM can interact with more than 300 enzymes in the cell. But the mechanisms for most of them are not clear yet, so there is a strong need to understand the binding mode between CaM and these target molecules. In Chapter 6, we use RyR1 as an example to determine the binding modes of CaM with its target enzymes. Three potential binding sites were observed in RyR1 and one of them was well proved. We confirmed the binding of CaM with RyR1\(_{3614-3643}\) peptide in both the presence and absence of Ca\(^{2+}\). We found that both N- and C- terminal domains contribute the binding with the peptide. However, only C –terminal domain bond to the peptide when CaM was in an apo form. These data tell us that CaM bind to the RyR1 in a different mode for apo- and holo- forms. \(^{15}\)N- labeled HSQC spectra also indicated that the two domains of CaM involved in the binding with another region in the channel which is RyR1\(_{1975-1999}\) in the presence of Ca\(^{2+}\). RDC data suggested that the apo CaM binds to RyR1\(_{3614-3643}\) peptide in the same mode as the IQ motif of mysin V-2IX7. A mini domain consists of both these
two regions was created to further determine the binding properties of CaM with the ryanodine channel and this will be a promising study as a future work.

10.5. Engineering CaM as a MRI contrast agent.

Magnetic resonance imaging (MRI) is a powerful diagnostic tool which is able to provide detailed information about the structure and composition of tumors. The use of exogenously administered contrast agents allows compartment-specific enhancement of diseased tissues by changing local proton relaxation times. So there is a strong need to create novel contrast agent satisfying the criteria to generate high quality images and targeting special cancers. We have reported the development of protein-based MRI contrast agents (ProCA1, previously named as CA1.CD2) by rational design of Gd$^{3+}$ binding sites into a stable protein using amino acid residues as metal coordinating ligands. CaM exhibit strong binding affinity with Gd$^{3+}$ and high physiological stabilities. It also provides high relaxivities (~ 20 times higher than Gd – DTPA) and large water numbers as described in Chapter 7 and 8. Based on these properties, we further engineered CaM as a MRI contrast agent candidate by fusing an affibody peptide which specifically target to HER-2 receptor and modified it by PEGylation to reduce the immunogenicity (ProCA22). ProCA22 was injected into the mice with xenograft tumor model. The mice were scanned on MRI instrument and tumor enhanced images were achieved. All the results indicate that our designed ProCA22 has the promising potential to be optimized as a protein based contrast agent.
References


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