Role of Neurogranin in the regulation of calcium binding to Calmodulin

Anuja Chandrasekar

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Role of Neurogranin in the regulation of calcium binding to Calmodulin

Anuja Chandrasekar, B.S

Advisor: M. Neal Waxham, Ph.D

The overall goal of my project was to gain a quantitative understanding of how the interaction between two proteins neurogranin (RC3) and calmodulin (CaM) alters a fundamental property of CaM. CaM, has been extensively studied for more than four decades due to its seminal role in almost all biological functions as a calcium signal transducer. Calcium signals in cardiac and neuronal cells are exquisitely precise and enable activation of some processes while down-regulating others. CaM, with its four calcium binding sites, serves as a central component of calcium signaling in these cells. It is aided in this role as a regulatory hub that differentially activates targets in response to a calcium flux by proteins that alter its calcium binding properties.

Neurogranin, also known as RC3, is a member of a family of small neuronal IQ (SNIQ) domain proteins that was originally thought to play a ‘capacitive’ role by sequestering CaM until a calcium influx of sufficient intensity arrived. However, based on earlier work in our lab on neurogranin, we believe that this protein plays a more nuanced role in neurons than simply acting as a CaM buffer. We believe that neurogranin is one of the proteins which, by altering the kinetics of calcium binding allow CaM to decode a variety of signals with fine precision. To quantify the interaction between CaM, neurogranin and calcium, I used biophysical techniques and computational simulations. From my results, I conclude that neurogranin finely regulates the proportion of calcium-saturated CaM and thereby directs CaM’s target specificity.
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ABBREVIATIONS

AMPA- α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (a glutamate receptor agonist)

Arg- Arginine, an amino acid

Asp- Aspartate, an amino acid

BICKS- B-50 immunoreactive C-kinase substrate

CA1- a region of the hippocampus

CA3- a region of hippocampus

CaCl₂ – Calcium chloride

CaM – Calmodulin

CaMKII- calcium/ CaM Kinase II

cDNA- complementary deoxyribonucleic acid

CHO- Chinese Hamster Ovary cells

COS- Fibroblast like cell line derived from monkey kidney tissue

CREB- cAMP responsive element binding protein

Cys- Cysteine, an amino acid

D- Aspartate, an amino acid

DTT- Dithiothreitol
EGTA- ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (a chelating agent)

ERK/MAPK- Extracellular signal-regulated kinases/Mitogen-activated protein kinase

G- Glycine, an amino acid

GAP-43- a SNIQ protein

HEPES- 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (a buffer)

IPTG- Isopropylthio-β-galactoside

ITC- Isothermal Titration Calorimetry

KCl- Potassium chloride

K_d – Dissociation constant

K_eq – Equilibrium constant

KO- Knockout

k_{off}- Off- rate of calcium dissociation

L-LTP- Long-long term potentiation

MOPS- 4-Morpholinepropanesulfonic acid (a buffer)

Ng- Neurogranin, a SNIQ domain protein

NIH-3T3- Fibroblasts in cell culture

NMDA- N-methyl D-aspartate (a glutamate receptor agonist)
ODE- Ordinary Differential Equation

PDE- Phosphodiesterase

PEP-19- a SNIQ protein

pET23- a plasmid

PMSF- Phenylmethanesulfonylfluoride

Quin-2- a calcium dye

SDS- Sodium dodecyl sulfate

Ser- Serine, an amino acid

skMLCK- skeletal myosin light chain kinase

SNIQ- Small Neuronal IQ domain proteins

TnI- troponin I

Tris- a buffer

UTR- Untranslated region

WT- Wildtype

ΔG- Free energy of interaction

ΔH- Enthalpy change

ΔS- Entropy change
CHAPTER 1

INTRODUCTION
Overview

I have divided this introduction into two sections. In the general introduction, I have attempted to provide a historical overview of CaM and neurogranin, their expression pattern, structure and function. In the introduction to concepts, I introduce the reader to fundamental principles that underlie the measurements and calculations used in my project and also provide a larger context of the biological significance of these proteins and the need to study them in such detail.

Section 1: General Introduction

1.1 Calmodulin (CaM)

1.1.1 CaM-Historical Perspective

Calmodulin (CaM) is the best-studied prototype of the EF-hand family of calcium sensing proteins. It is a 148 amino acid protein that was first discovered in brain and heart as an activator of cyclic nucleotide phosphodiesterase and isolated using a gel filtration column ((Kakiuchi et al. 1975), (J. H. Wang et al. 1975), (Teo and Wang 1973), (Cheung 1970)). This cytosolic protein is the primary calcium sensor in almost every cell in every eukaryotic organism studied. The functions of CaM are diverse ranging from regulation of various stages of the cell cycle to transcriptional regulation in the nucleus to regulation of synaptic plasticity in neurons.

In neurons, CaM interacts with kinases like calcium/CaM dependent protein kinase I, II and IV (CaMK I,II and IV), the phosphatase calcineurin, small neuronal IQ domain
proteins (SNIQ) proteins like neuromodulin, neurogranin, PEP-19, enzymes like nitric oxide synthase and ion channels like L-type calcium channels, sodium channels, potassium channels to name a few (Prichard, Deloulme, and Storm 1999, Peterson et al. 1999, Kim et al. 2004, Putkey et al. 2003, Zhang et al. 1994, Mulkey et al. 1994). Interestingly, many of these CaM binding proteins like neuromodulin, neurogranin, PEP-19, L-type calcium channels and sodium channels have an IQ domain through which they interact with CaM. This raises the interesting possibility that studying IQ domain proteins might provide valuable insights into regulation of CaM and help answer fundamental questions of calcium metabolism in the brain.

1.1.2 CaM expression

The vertebrate CaM family is a unique example of genetic redundancy where multiple non-allelic genes encode the identical protein and not isoforms of each other. CaM is a highly conserved protein and is encoded by three different genes in mammals, CaM I, II and III (Nojima 1989). All three CaM genes are expressed in neurons and their levels of expression are differentially regulated (Gannon and McEwen 1994). For example administration of NMDA antagonist AP-5 to rats resulted in specific reduction of CaM I mRNA in the caudal colliculus at postnatal day 10 (Berry, Prusky, and Brown 1996). Treatment of adult mice with kainate led to an increase in CaM I mRNA, a decrease in CaM II mRNA and no change in CaM III expression (Michelhaugh, Pimputkar, and Gnegy 1998). CaM expression is likely strictly regulated in neurons because of its involvement in a variety of neuronal functions. The redundancy of genes encoding the identical protein (unique to CaM) and the strict regulation of CaM translation indicate that there are multiple regulatory levels to ensure accurate CaM expression. This tight regulation
of CaM protein expression is evidence of the importance of this protein in biologic processes.

1.2 Structure of CaM

1.2.1 Crystal structure

The crystal structure of CaM was first determined at 3Å resolution (Babu et al. 1985) and subsequently at 3.6Å by (Kretsinger, Rudnick, and Weissman 1986). The structure was further refined at 2.2 Å and 1.7 Å by two different groups (Babu, Bugg, and Cook 1988, Chattopadhyaya et al. 1992). The CaM structure is dumbbell shaped, with an overall length of 65 Å. Each lobe is 25x20x20 Å and connected by an eight-turn α-helix. Each CaM molecule has two EF hand domains (calcium-binding loops) arranged in a globular N-terminal domain followed by a central helix linker and two EF hand domains arranged in a globular C-terminal domain. Adjacent calcium-binding loops are separated by double-stranded antiparallel beta-sheets. The N-terminal globular domain has a lower affinity of calcium binding and a faster off-rate and on-rate. The C-terminal globular domain has a higher affinity towards calcium and slower off-rate and on-rate. Upon binding calcium, CaM is activated and in turn recognizes protein targets.
Figure 1.1: Crystal structure of apoCaM and calcium/CaM

Crystal structure of apoCaM (left) and calcium/CaM (right). Calcium binds to the two EF hand loops in the N-terminal lobe and C-terminal lobe. The region at the center of the helix (unwound in apo-CaM) is believed to undergo changes in solution and upon binding to targets.
1.2.2 Solution structure

The solution structure of CaM was studied in 1988 by Heidorn and Trehella (Heidorn and Trehella, 1988). They concluded based on modeling studies and X-ray solution scattering data that there is a difference between the crystal structure and the solution structure and that the solution structure involves considerable structural changes in the helix connecting the globular domains. Furthermore, structural changes upon target binding to CaM in solution was studied by Ikura and colleagues (Ikura et al. 1992). This group studied calcium ligated CaM complexed with skMLCK peptide. They discovered that the residues aspartate 78 through serine 81 which are located in the middle of the ‘central helix’ of CaM forms a very flexible link between globular domains. They concluded that the helical skMLCK peptide is located in a hydrophobic channel going through the center of the complex and makes an angle of ~45° with the long axis of the complex.

1.2.3 Association of CaM’s structural flexibility and calcium transduction functions

A fascinating aspect of CaM biochemistry is its ability to respond to calcium signals of different frequency and amplitude and activate different targets. A study of the structure of CaM shows that this remarkable function might be tied to its structural flexibility. Multidimensional heteronuclear nuclear magnetic resonance spectroscopy of structure of C-terminal of CaM in solution indicated major rearrangements within the secondary structure elements with changes in inter-residue distances of up to 15 Å. This structural rearrangement within each globular domain results in exposure of the hydrophobic residues within a methionine-rich crevice in each domain that is distinct from the calcium binding loop. The exposure of hydrophobic residues to solvent is equivalent to a calcium-controlled
unfolding of CaM and releases free energy. It is this ability to convert a biochemical event into free energy that forms the basis of CaM’s ability to transduce calcium signals (Finn et al. 1995).

1.3 Functions of CaM

CaM through its ability to bind to calcium ions regulates a number of important biological functions like cell growth, gene expression, cell motility, protein phosphorylation-dephosphorylation cascades, ion transport, calcium transport, cytoskeletal function and neurotransmitter and hormone action to name a few ((Youn et al. 1999; Rasmussen and Means 1989; Hinrichsen and Blackshear 1993)).

1.3.1 Role of CaM in long-term potentiation in neurons

CaM plays an active role in regulating a plethora of diverse and important neuronal functions. Perhaps, the best characterized and the most well-studied function of CaM in neurons is its role in long-term potentiation and long-term depression.

Long-term potentiation was first described by Bliss and Lomo in the hippocampal formation (Bliss and Lomo 1973). When a synapse is repeatedly stimulated, transmission of signal between the two neurons at that synapse is strengthened. One of the reasons for strengthening is believed to be recruitment of additional neurotransmitter receptors at the synapse as well as modifications to the synaptic receptors which increase ion influx through them. Calcium/CaM activates the multifunctional protein CaMKII. At resting calcium, CaMKII is auto-inhibited. When calcium rises, binding of Ca$^{2+}$/CaM to CaMKII relieves inhibition and the activated
CaMKII phosphorylates AMPA receptors as well as other targets which play a role in long-term potentiation (Malenka, R.C. et al., 1989; Malinow, R et al., 1989)

1.3.2 CaM and long-term depression in neurons

While CaM responds to high calcium influx by activating CaMKII, in response to low calcium influx, CaM activates calcineurin. Long-term depression is a reduction in strength of synaptic transmission following constant, low frequency stimulation that was first reported in cerebellar cortex by Ito and Kano (Ito and Kano, 1982). Calcineurin is a calcium-dependent serine-threonine phosphatase that has been implicated in long-term depression. This protein is activated by CaM whereupon it dephosphorylates and inactivates inhibitor-1 which in turn increases protein phosphatase 1 and contributes to long-term depression (Mulkey et al. 1994)

1.3.3 CaM involvement in hippocampal long-long term potentiation

In the hippocampus, long-long term potentiation (L-LTP), a type of long-term potentiation at the CA1-schaffer collateral synapse is dependent on de novo transcription and translation. CaM is not the primary molecular player involved in L-LTP but acts through activation of proteins involved in L-LTP. Activity dependent translation at the synapse is initiated by activated cAMP responsive element binding protein (CREB) (Barco, Alarcon, and Kandel 2002). CREB is activated by phosphorylation by three main enzymes - ERK/MAPK, adenylyl cyclase and CaMKIV –all of which are regulated by CaM.
1.3.4 CaM's role in regulation of calcium influx into neurons

CaM, besides regulating calcium dynamics within the neuron leading to long-term potentiation and long-term depression also controls calcium influx into the neuron. It performs this role by binding constitutively to some channel proteins through IQ-like motifs present in the intracellular domains of these channels.

For instance, CaM was found to be crucial for calcium-dependent inactivation of L-type calcium channel (LTCC). Expression of mutants of CaM (CaM (3-)) incapable of binding calcium resulted in loss of calcium-dependent inactivation (Zühlke et al. 1999). The importance of CaM for calcium-dependent inactivation was further reinforced by David Yue and colleagues when they overexpressed calcium-insensitive CaM and found that this ablated calcium-dependent inactivation in a “dominant negative” manner (Peterson et al. 1999). CaM was also found to be important for functional expression of some isotypes of voltage gated sodium channels (Na,v1.4 and Na,v1.6), and impacted the inactivation kinetics and the current density through the channel (Herzog et al. 2003). Both these channels regulate calcium influx into neurons.
Table 1.1: Examples of CaM-activated proteins that regulate a variety of biological functions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Function of protein</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cabin1</td>
<td>Thymocyte transcriptional regulator</td>
<td>Youn, H.D. et al., 1999</td>
</tr>
<tr>
<td>NAP-22</td>
<td>Neuronal substrate of protein kinase C</td>
<td>Yamamoto, Y et al., 1997</td>
</tr>
<tr>
<td>Striatin</td>
<td>Neuronal protein that associates with phosphatase 2A</td>
<td>Moreno, C.S et al., 2000</td>
</tr>
<tr>
<td>Phosphatidyl inositol 3-kinase</td>
<td>Component in receptor signaling from GPCR</td>
<td>Joyal, J.L et al., 1997</td>
</tr>
<tr>
<td>L-type calcium channels</td>
<td>Voltage gated calcium channel in neurons</td>
<td>Erickson, M.G. et al., 2001</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Kinase implicated in long-term potentiation in the brain</td>
<td>Malenka, R.C. et al., 1989</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Malinow, R. et al., 1989</td>
</tr>
<tr>
<td>Calcineurin</td>
<td>Phosphatase involved in long-term depression in the brain</td>
<td>Mulkey, R.M. et al., 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Mulkey, R.M. et al., 1994</td>
</tr>
</tbody>
</table>
1.4 Post translational modifications of CaM and its effect on target binding capabilities of CaM

There has been significant research on the post-translational modifications of CaM targets and the functional implications on CaM-target recognition. Following are examples of post-translational modifications of CaM and their functional implication.

1.4.1 Methylation

CaM is methylated at lysine 115 in higher organisms. However, the functional implication of methylation is still unclear as unmethylated CaM can activate most known target proteins except NAD kinase which is more sensitive to activation by unmethylated CaM (S.-H. Oh and Roberts 1990; S. H. Oh et al. 1992; Bitensky 1988).

1.4.2 Glycation

Glycation of lysine side chains of CaM reduces CaM’s ability to bind to calcium and activate target enzymes. Although glyco-CaM is not normally found, it has been isolated from diabetic individuals and maybe involved in the histopathological development of the disease(Kowluru et al. 1989).

1.4.3 Phosphorylation

CaM is phosphorylated at multiple sites. Phosphorylation has specific effects on target activation and hence represents a mechanism by which CaM differentially regulates targets within a cell. Phosphorylation of CaM by Casein Kinase II reduced the binding affinity of CaM for PDE without changing the activation kinetics(Sacks et al. 1992). Phosphorylation at tyrosine 99 generated a form of CaM that more effectively activated PDE and had a greater affinity for this target than the wild-type CaM.
1.5 Small neuronal IQ (SNIQ) domain proteins - a target of CaM

Small neuronal IQ domain proteins also known as calpacitins are a family of proteins that have an IQ domain through which they interact with CaM. The name ‘calpacitin’ was based on the observation that members of this protein family act like capacitors, binding to CaM at resting conditions and releasing it slowly or rapidly depending on the amplitude and frequency of calcium flux (Gerendasy and Sutcliffe 1997; Gerendasy et al. 1994). These proteins are characterized by their sequence homology in and around their CaM-binding domain, high concentrations within specific regions of the neuron as well as their ability to interact with calcium-free and calcium-bound forms of CaM in a regulated manner allow these proteins to regulate the calcium binding kinetics of enzymes that require Ca\(^{2+}\)/CaM.

1.5.1 Members of SNIQ domain protein family

The well-studied members of the family of SNIQ domain proteins are PEP-19 also known as Pcp 4, GAP-43 also known as neuromodulin and RC3 also known as neurogranin. Two of these proteins are relatively small in size. PEP-19 is a 61-amino acid polypeptide and neurogranin is 78-amino acid long. Neuromodulin is a slightly larger protein that is 239 amino acids long. With the exception of PEP-19 interactions between CaM and each of the calpacitins can be regulated by post-translational modifications. For example, neurogranin and neuromodulin contain a serine residue within their CaM-binding domain that can be phosphorylated and whose phosphorylation abrogates CaM binding to these proteins (Huang et al. 2004). In addition, nitric-oxide induced oxidation of neurogranin has been shown to attenuate its affinity for CaM (Li et al. 1999; Mahoney, Pak, and Huang 1996; Sheu et al.
This results in additional regulatory mechanisms that can finetune CaM-calcium
interaction. The post-translational modifications of neurogranin are described in detail in the
next section where I use it in discussing the biological significance of neurogranin.

1.5.2 Expression patterns of SNIQ proteins

Neuromodulin is predominantly expressed in the neocortex, areas CA1 and CA3 of
the hippocampus, subcortical nuclei such as the locus coeruleus, inferior olivary nucleus and
part of the thalamus and hypothalamus(Benowitz et al. 1988; McNamara and Lenox 1997;
Kruger et al. 1993). Neurogranin is expressed in the cortex, hippocampus and striatum with
lower levels of expression in the olfactory bulb and midbrain and no expression in the
cerebellum(Watson, Szijan, and Coulter 1994). PEP-19 is expressed in several regions of the
nervous system including the cortex, putamen, olfactory bulb, retina, substantia nigra,

1.5.3 Role of SNIQ proteins in neurons

The function of small neuronal IQ (SNIQ) domain proteins in neurons is of interest
because of the high concentrations of these proteins in neurons (Ziai et al. 1986, Prichard,
Deloulme, and Storm 1999). In fact, many members of this protein family were first isolated
from various regions of the brain. PEP-19 was first discovered in functional genomics
studies that aimed to identify markers of cerebellar development. One of the ways
neurogranin was discovered was using a subtractive hybridization screen that aimed to
identify proteins expressed in the cortex but not in the cerebellum(Prichard, Deloulme, and
Storm 1999). Neuromodulin was discovered as a protein whose phosphorylation increased
during the induction of synaptic plasticity(Akers and Routtenberg 1985).
a) **PEP-19**: PEP-19 expression in the cerebellum is widely considered to serve a protective function in Alzheimer’s Disease (AD). The primary afferent neurons in the cerebellum are the Purkinje cells where PEP-19 expression is at a high-level. These cells are mostly spared from the disease. However, PEP-19 negative pyramidal cells that are downstream of the granule cells in the dentate gyrus show considerable loss of neurons in Alzheimer’s disease (Slemmon et al. 1994). In Huntington’s Disease, the mutant huntingtin protein demonstrates an increase in affinity to CaM when compared to the wild-type huntingtin protein. This could potentially result in regulation of calcium homeostasis through altered interaction with CaM. Furthermore, structures affected in Huntington’s disease including caudate nucleus, putamen, globus pallidus, and substantia nigra show a large loss in PEP-19 expression (Utal et al. 1998). It is possible that PEP-19 loss accelerates the process by which calcium homeostasis is lost in Huntington’s disease.

b) **Neuromodulin**: Neuromodulin is a SNIQ domain protein concentrated in the growth cones of presynaptic neurons. This localization suggests that it is involved in axon elongation or in synapse organization and reformation. In toad retinal ganglion cells, neuromodulin synthesis began approximately four days after axotomy and the rise in neuromodulin production coincided with or slightly preceded initiation of axon outgrowth. Further evidence that neuromodulin might contribute to growth cone activity by regulating cell membrane structure came from experiments in CHO, COS and NIH 3T3 cells. Expression of neuromodulin in these cells resulted in the induction of filopodium-like structures projecting from the cytoplasmic membranes of these non-neuronal cell lines. (Liu and Storm 1990) proposed a biochemical model
where neuromodulin sequesters CaM at membrane tips. Phosphorylation of neuromodulin at its critical CaM binding domain by protein kinase C would release high concentrations of CaM locally in the growth cones. The release CaM interacts and affects the polymerization and crosslinking of polymers like actin and spectrin resulting in growth cone elongation. Thus various SNIQ domain proteins, through their interaction with CaM play an important role in neurons.

1.6 Neurogranin- a SNIQ protein

1.6.1 Historical perspective

Neurogranin, named because of its granular appearance in immuno-cytochemical studies, was first discovered in a subtractive hybridization screen looking for mRNAs expressed in the forebrain but not the cerebellum (Watson et al. 1990). The protein was termed RC3 for rodent cortex enriched clone #3. It was isolated a second time from bovine brain as a perchloric acid soluble substrate of protein kinase C and called p17 based on the apparent molecular weight of the protein on SDS gels (Baudier et al. 1991). It was discovered a third time based on its cross-reactivity to neuromodulin antibodies and was called B-50 immunoreactive C-kinase substrate (BICKS) (Coggins and Zwiers 1990).

Sequencing of neurogranin revealed that the protein sequence shared many homologous regions with neuromodulin. Neurogranin and neuromodulin show a high degree of homology around the IQ domain as well as in their N-termini. The homologous regions in neurogranin and neuromodulin includes the serine phosphorylation site and a peptide sequence that forms an efficient and specific PKC
substrate (the IQ motif). The region around the IQ motif with is involved in CaM binding and membrane-PKC interactions.

**Figure 1.2: Multiple protein sequence alignment of RC3 and GAP-43 sequences from rat, cow and humans**

<table>
<thead>
<tr>
<th></th>
<th>Rat RC3</th>
<th>Cow RC3</th>
<th>Human RC3</th>
<th>Rat GAP-43</th>
<th>Cow GAP-43</th>
<th>Human GAP-43</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MDCTESACS CPDPDDLPLDPPG ANAAAALKIQASFRGHRMHRKKKIKSREGGRKFGPQPQPQPQPQAAGG PSSG</td>
<td>MCTESACS CPDPDDLPLDPPG ANAAAALKIQASFRGHRMHRKKKIKSREGGRKFGPQPQPQPQPQAAGG PSSG</td>
<td>MDCTENACS CPDPDDLPLDPPG ANAAAALKIQASFRGHRMHRKKKIKSREGGRKFGPQPQPQPQPQAAGG PSSG</td>
<td>MLCMMRRTKQV DE D QK/eqeQG K VHAATKIQQASFRGHITRKKLQDKEK K GD APAAEAEAEAEKDKDAP ADG EKKE</td>
<td>MLCMMRRTKQV DE D QK/eqeQG K VHAATKIQQASFRGHITRKKLQDKEK K GD APAAEAEAEAEKDKDAP ADG EKKE</td>
<td>MLCMMRRTKQV DD D QK/eqeQG K VHAATKIQQASFRGHITRKKLQDKEK K GD APAAEAEAEAEKDKDAP ADG EKKE</td>
</tr>
</tbody>
</table>

**1.6.2 Genetics of neurogranin: Encoding genes**

The rat neurogranin cDNA isolated through subtractive hybridization encoded a 78 amino acid sequence product. Using antisera raised against peptides from two different regions of the translated product, neurogranin protein was detected in the forebrain by immunocytochemistry. The mouse homolog was then cloned and used to map the gene to the proximal region of chromosome 9 (Danielson et al. 1994). The locus was designated *Pss1* for post synaptic spine 1. The human neurogranin gene was mapped to chromosome 11q24 which is syntenic with the mouse locus *Pss1*. Northern blots of rat and mouse forebrain revealed mRNAs of two lengths 1.0 kb and 1.4 kb (Watson et al. 1990).

The human and rat genes have four exons and three introns. The first exon codes the 5’ untranslated region (UTR) and the first five residues of the protein. The second exon encodes the bulk of the protein (73 residues) and a part of the 3’ UTR while the third exon contains an 18 base pair portion of the 3’ UTR. The fourth exon, which has only 70% homology between rat and humans contains the rest of the 3’ UTR and the polyadenylation
signal. The first intron is long, about 5.5 kb in human, while the second and third introns are 233 bp and 408 bp respectively, in human.

**1.6.3 Expression pattern of neurogranin**

The mRNA is abundant in various thalamic and hypothalamic nuclei, several regions of the olfactory bulb and tubercle, the medial amygdaloid nucleus and pontine nucleus as well as in the caudate putamen, the CA1 and CA3 regions of the hippocampus, the dentate gyrus, the primary olfactory cortex, the somatomotor cortex and the frontal cortex. The protein expression is more restricted and is abundant in the frontal parietal cortex, granular cells of the dentate gyrus, apical dendrites of pyramidal cells of the CA1 and CA3 regions of the hippocampus and in the striatal cortex.

The peak expression of the protein occurs between postnatal day 14 and postnatal day 20 (Alvarez-Bolado et al. 1996; Represa et al. 1990) which is the period during which more than 80% of cortical synapses are formed. Interestingly, an increase in neurogranin expression coincides with the onset of synaptogenesis in rats and mice and its expression occurs in those regions of the brain that exhibit a high degree of neuroplasticity ((Hashimoto et al. 1988; Sposi et al. 1989) providing a developmental neurobiology argument for neurogranin’s contribution to neuroplasticity.
Section 2: Introduction to concepts

1.7 Thermodynamic principles used in study

1.7.1 Free energy of coupling

When two molecules interact, there is a change in free energy. In 1982, David Storm’s group at University of Washington, investigated this change in free energy for the interaction between calcium, CaM and a CaM-binding protein troponin I using fluorescence measurements and equilibrium dialysis. The aim was to characterize the binding of calcium to CaM in the presence of CaM-binding protein. The data obtained from the experiments was analyzed using the method proposed by Weber (Weber 1975).

Figure 1.3: Scheme for interaction between CaM and troponin-I

In the scheme, TnI refers to Troponin I, ΔG°(C) refers to the free energy change upon binding of 1 mol of calcium to CaM, ΔG°(I) stands for the free energy change upon binding of TnI to CaM, ΔG°(C/I) is the free energy change upon binding of 1 mol of
calcium to the CaM-TnI complex and $\Delta G^\circ(I/C_4)$ is the free energy change upon binding of TnI to fully saturated CaM.

Conservation of free energy requires

$$4[\Delta G^\circ(C/I) - \Delta G^\circ(C)] = \Delta G^\circ(I/C_4) - \Delta G^\circ(I) = 4\Delta G^\circ_{IC}$$

where $\Delta G^\circ_{IC}$ is the average free energy of coupling for binding of 1 mol of calcium and troponin I to CaM. Based on calculations of $\Delta G$ from experimentally measured $K_d$, it was estimated that the affinity of troponin I for CaM is increased 4500 fold when CaM is saturated with calcium. This concept of free energy and calculation of free energy from dissociation constants is important for this dissertation because I have used these concepts in evaluating the quantitative interactions of neurogranin with CaM in the absence and presence of calcium.

1.7.2 Conservation of free energy

Another thermodynamic principle of importance in my work is the idea that the free energy of binding ($\Delta G$) of a chain of binding reactions should be the same regardless of the order of binding. Illustrated below is a scheme of binding of calcium and target to CaM. The free energy to go from apo-CaM to target-bound fully saturated CaM is the same irrespective of whether calcium binds first or the target binds first.

Using the example below,

$$\Delta G_1 + \Delta G_2 + \Delta G_7 = \Delta G_5 + \Delta G_{12} + \Delta G_{11}$$
Since,

$$\Delta G = -RT \ln(K_d)$$

$$K_{d1} * K_{d2} * K_{d7} = K_{d5} * K_{d12} * K_{d11}$$

$$K_{eq1} * K_{eq2} * K_{eq7} = K_{eq5} * K_{eq12} * K_{eq11}$$

If $K_{eq11}$ is higher than $K_{eq1}$, $K_{eq2} * K_{eq7}$ should be higher than $K_{eq5} * K_{eq12}$ to maintain equality.

Since $K_{eq}$ is a measure of affinity of interaction, the implication is that if a target binds to calcium/CaM with higher affinity, the probability of target bound CaM remaining fully saturated with calcium is higher.
Figure 1.4: Illustration of the principle of conservation of free energy

(Brown and Bertolet, 1997)
1.8 Kinetics of calcium binding to CaM

The versatility and tenability of CaM has spawned interest in the kinetics of calcium binding for decades. Multiple groups have studied the kinetics of calcium binding to CaM under different experimental conditions and using fragments of CaM as well as the whole molecule.

Peter Bayley and colleagues used stopped flow fluorescence to measure calcium dissociation rates from the N-terminal and C-terminal lobes of CaM. They determined the rate of calcium dissociation from the N-terminal lobe to be 293 to 550 s\(^{-1}\) for a temperature range of 11 to 19\(^{\circ}\)C. The rate of calcium dissociation from the C-terminal lobe was determined to be 2 to 9 s\(^{-1}\) for a temperature range of 11 to 28\(^{\circ}\)C.

In order to determine calcium dissociation rates of individual binding sites, Linse and co-workers used tryptic fragments to measure calcium dissociation rates from fragments which contained only the N-terminal domain calcium binding sites or C-terminal calcium binding sites. They determined that the macroscopic binding constants of the tryptic fragments are identical to the sites in intact CaM (\(K_{D1} = 12.6 \text{ M}\), \(K_{D2} = 0.25 \text{ M}\) in the C-terminal lobe, and \(K_{D3} = 40 \text{ M}\), \(K_{D4} = 2.5 \text{ M}\) in the N-terminal lobe). Putkey and colleagues reported values for equilibrium constants of calcium binding which were very similar to those reported by Linse and colleagues (Putkey et al., 2008).
1.9 Significance of studying calcium transduction by CaM

The calcium binding properties of CaM have been of interest to researchers for decades. The three main factors that make CaM such a key molecular player in calcium signal transduction are its abundance and ubiquitous presence, its versatility as a calcium sensor and its tunability by targets.

CaM is ubiquitously and abundantly present in many cells and plays a critical role in regulating the biological function of these cells. For example, in the central nervous system CaM is present in extremely high concentrations (from 10 to 100 uM in different areas of the brain (Cimler et al. 1985) and transduces calcium signals following an action potential.

The four calcium binding sites of CaM bind calcium with different affinities and different rates of binding. This property allows CaM to act as a versatile calcium sensor that can transduce calcium signals of different frequency and amplitude (Bayley et al. 1984; Teleman, Drakenberg, and Forsén 1986; Linse, Helmersson, and Forsén 1991).

Finally, there are more than 300 targets of CaM that have been identified. Many of these targets of CaM interact with it reciprocally and alter the affinities and rates of calcium binding making CaM a tunable calcium sensor.
1.10 Quantification of reciprocal effect of targets on CaM

The ratio of CaM targets to CaM has been estimated to be 2:1 by some groups (Slemmon and Martzen 1994; Slemmon and Flood 1992; Slemmon et al. 1996). From a mass action standard point, this estimate of concentration ratios makes the regulation of CaM by its targets a very attractive hypothesis. Quantitation of the interaction between CaM and its targets provides good insights into the regulation of CaM’s properties by its targets. Explained below are two examples of methods by which CaM’s interaction with targets and the reciprocal effect of targets on CaM’s calcium interaction have been accurately quantified using quantitative experimental techniques.

Olwin and colleagues determined free energies of coupling for the interaction of calcium and myosin light chain kinase with CaM by micro-equilibrium dialysis using $^{45}$CaCl$_2$ and by fluorescence anisotropy using fluorescent labeled CaM (Olwin et al., 1984). The mean dissociation constants for calcium interaction with CaM in the presence and absence of enzyme were 0.40 and 14 µM, respectively, yielding a minimal estimate for free energy of coupling of -2.11 kcal/mol of Ca$^{2+}$ (-8.44 kcal/4 mol of Ca$^{2+}$). The free energy coupling for binding of myosin light chain kinase to CaM and substrates was -0.95 kcal/mol of CaM. From this data, the interpretation was that in the presence of myosin light chain kinase interaction of calcium with CaM was a highly ‘cooperative’ process.

Another example where equilibrium and kinetic constants were used to determine interaction of CaM and its targets was the study of CaM interaction with endothelial nitric oxide synthase and a fluorescent myosin light chain kinase (MLCK) analog. The $K_d$ and $K_{on}$ of nitric oxide synthase for Ca$^{2+}$/CaM are 0.2 nM and 1.5 x $10^5$ M$^{-1}$s$^{-1}$ which are 10 and 100
fold smaller than the values for the MLCK analog. Thus, even though the target MLCK analog binds CaM quickly due to its high association rate constant, it dissociates quickly from CaM as well due to the high dissociation rate. At equilibrium, a greater percentage of CaM is expected to bind to nitric oxide synthase since it has a higher affinity to CaM. As expected, when calcium was added to a mixture of CaM, target analog and synthase in vitro, a large fluorescent transient corresponding to a rapid binding of analog to calcium/CaM followed by a slower capture of calcium/CaM by higher affinity synthase was seen.

1.11 Neurogranin as a CaM target

Neurogranin was discovered as a protein kinase C (PKC) substrate isolated from mouse hippocampi. The biochemical properties of neurogranin like its ability to regulate CaM-dependent nitric oxide synthase, its interaction with membrane phospholipids and its phosphorylation by PKC implicated it in several signaling pathways (Martzen and Slemmon 1995; Slemmon and Martzen 1994; Lu and Chen 1997; Paudel, Zwiers, and Wang 1993). However, the physiological functions of neurogranin was unclear. Storm and colleagues utilized yeast two-hybrid technology to detect neurogranin-binding proteins \textit{in vivo} (Prichard, Deloulme, and Storm 1999). They determined that the only protein that neurogranin interacted with \textit{in vivo} was CaM. Detailed biochemical studies on neurogranin by Gerendasy and group led them to suggest that neurogranin had a non-linear response to calcium, releasing CaM rapidly in response to a large calcium influx and slowly in response to small increases analogous to the behavior of a capacitor. However, systematic studies on the interaction between neurogranin and CaM using stopped flow fluorimetry and fluorescence anisotropy suggested that neurogranin also tunes CaM’s calcium binding
properties. Furthermore, post-translational modification of neurogranin tuned neurogranin’s ability to interact with CaM.

These properties of neurogranin led our group to quantify the interaction between neurogranin and CaM.

1.12 Post-translational modifications of neurogranin

1.12.1 Phosphorylation and de-phosphorylation of neurogranin

The primary amino acid sequence of neurogranin has some highly conserved residues which are homologous to a region near the amino acid terminus of GAP-43. This conserved stretch of residues contains the site of phosphorylation by protein kinase C (Baudier et al. 1991) which is a serine at position 36. The ability of neurogranin to be phosphorylated is critical for its role in long-term potentiation (Ramakers et al. 1995). Phosphorylation of RC3 interferes with its binding to CaM. Conversely, interaction of CaM with RC3 can inhibit phosphorylation by PKC, presumably by blocking access of the kinase to serine 36. Neurogranin, by virtue of being a PKC substrate is ideally situated to be at the intersection of the two signaling pathways from the metabotropic and glutamergic receptor pathways.

Neurogranin is dephosphorylated \textit{in vitro} by calcineurin and protein phosphatases 1 and 2A (Seki \textit{et al.}, 1995). This raises the interesting possibility that neurogranin, like CaM is subjected to the influence of both kinases and phosphatases and the amount of activated neurogranin might be finely controlled by the balance between activation by phosphorylation and inactivation by dephosphorylation.
1.12.2 Other post-translational modifications of neurogranin

Nitric oxide causes oxidation of neurogranin in rats. Neurogranin has four cysteine residues at positions 3, 4, 9 and 51. Systematic replacement of each of the cysteine residues individually or in combinations with serine and glycine and examination of the intramolecular disulfide bonds formed indicated that cysteine 51 forms a disulfide bond that is induced by oxidants (Mahoney, Pak, and Huang 1996). The disulfide bond between cysteine 51 and any of the other cysteine residues at positions 3, 4 or 9 presumably alters the conformation of the PKC-recognition/CaM-binding domain that lies between Cys51 and the other three cysteine residues.

Neurogranin was found to bind selectively to phosphatidic acid when lipid-protein overlay assays were done using PIP strips. Furthermore, interaction of neurogranin and phosphatidic acid is prevented by CaM and inhibited by PKC phosphorylation of the serine residue in the IQ domain. This is an interesting result because phosphatidic acid is a membrane lipid and it is believed that interaction with this lipid might serve to localize neurogranin to membranes. Phosphorylation might thus serve to disrupt membrane localization of neurogranin apart from interfering with its interaction with CaM.

1.13 Neurogranin and synaptic plasticity

LTP induction in hippocampal extracts when led to an observed increase in post hoc phosphorylation of a 17 KDa PKC substrate which was later named neurogranin (Klann, Chen, and Sweatt 1992; Klann, Chen, and Sweatt 1993). Long term potentiation (LTP) is a phenomenon where when a synapse is repeatedly stimulated, transmission of signal between the two neurons at that synapse is strengthened. The findings in hippocampus were
consistent with other *in situ* phosphorylation studies that demonstrated neurogranin phosphorylation when LTP was induced (Gianotti et al. 1992; Ramakers et al. 1995).

Neurogranin’s role in LTP was further demonstrated in experiments conducted by Fedorov and colleagues in which a monoclonal antibody introduced against the CaM binding/PKC recognition domain of neurogranin in hippocampal CA1 region blocked LTP (Fedorov et al. 1995). Quantitative immuno-precipitation of neurogranin 10, 30, 60 and 120 minutes after LTP induction lead to the observation that there was a 78% increase in phosphorylation only at the 60 min time-point (Ramakers et al. 1995). Phosphorylation was NMDA-dependent and did not occur in those slices that failed to produce LTP.

### 1.14 Neurogranin knock-out mice

Neurogranin knock-out mice were generated by Gerendasy and colleagues and Huang and colleagues. In both cases, neurogranin was knocked-out by homologous recombination. These mice did not show any gross developmental or neuroanatomical abnormality. Studies in neurogranin knock-out mice revealed that calcium influx through NMDA receptor was lower in the case of neurogranin knockout mice compared to wildtype mice (van Dalen et al. 2003). Lowered calcium influx could result in reduced activation of calcium dependent proteins that are required for LTP. Furthermore, in another study, neurogranin knockout mice showed deficiencies in hippocampal short and long-term plasticity as seen by their performance in the morris water maze test, an assay to test spatial memory. These mice also displayed decreased basal level of activated calcium/CaM kinase II which is a protein crucial for induction of LTP (CaMKII in knock-out mice ~ 60% of wildtype mice) (Pak et al. 2000).
Interestingly, the knockout mice from Gerendasy and group showed an enhancement in LTP induction and a reduction in LTD induction. These results seem to be contradictory to the results published by Pak and colleagues. Thus, knocking out neurogranin seems to affect the degree of synaptic potentiation which is likely due to the regulation of CaM by neurogranin.

1.15 Crystal structure of neurogranin bound to CaM

In March 2013, the crystal structure of a region of neurogranin bound to CaM was determined by Gerges and group (Kumar et al. 2013). This was the first successful attempt at crystallization of a region of neurogranin. Initial attempts at crystallizing the full protein were unsuccessful because neurogranin is an intrinsically disordered and unstructured protein that is not amenable to crystallization. Hence, Gerges and colleagues used a shorter peptide sequence of neurogranin containing mostly the CaM binding IQ domain and a few amino acids outside of the IQ domain, RC3(27-50). They linked this peptide to the C-lobe of CaM using a 5 glycine linker and crystallized this CaM-bound structure.

The crystal structure of RC3(27-50) showed that the neurogranin peptide adopted an α-helical structure that was almost perpendicular to the central α-helix of CaM, making several contacts with the C-lobe and a few contacts with the central helix of CaM. The amino acid residues within the IQ region that interact with CaM significantly are Ile33, Ser36 and Arg38. Serine 36 phosphorylation disrupts binding to CaM and phosphatidic acid. This residue of neurogranin in the crystal structure was buried in a pocket surrounded by negatively charged residues of CaM. Phosphorylation of the serine residue likely repels the negative charges in the pocket weakening binding to CaM. Arginine residue which is highly
conserved and is another residue which was found to form contacts with CaM might have an essential role in mediating the interaction of IQ motif with CaM. A mutation of this residue to glutamine reduced the binding strength of neurogranin to CaM.

In summary, the first reported crystal structure of the intrinsically disordered protein neurogranin in complex with CaM provides a structural basis for the interaction of neurogranin with CaM, a crucial association for several learning and memory-associated functions in neurons.
Figure 1.5: RC3 peptide interaction with the C-lobe of CaM
CHAPTER 2
MATERIALS AND METHODS
2.1 Isothermal Titration Calorimetry (ITC)

Isothermal titration calorimetry (ITC) is a technique that measures the endothermic/exothermic change when molecules interact. The ITC machine used to make measurements is the VP-ITC machine from microcal (GE healthcare systems). The experiment consists of injection of a small volume of one sample containing one of the interacting molecules into a sample cell containing a solution of the other molecule. There is also a reference cell filled with water/buffer in which the solutions are made. Each injection occurs over a fixed time period with a fixed time interval between consecutive injections. The readout from the ITC machine is the difference in temperature between the reference and sample cell calibrated to power units. The area under the curve for each injection is integrated and the resulting data points are fit using a binding curve from which the dissociation constant $K_d$ is calculated.

Most of the ITC experiments were carried out at 25°C and a few experiments were carried out at 37°C. In the case of neurogranin peptides, the peptides are present at a concentration of 150 µM in a buffer solution of 25 mM HEPES or a solution containing 25 mM HEPES and 150 mM KCl in the syringe. The sample cell contains the protein at a concentration of 10 µM in a buffer solution of 25 mM HEPES or a solution containing 25 mM HEPES and 150 mM KCl. Each injection occurs over a duration of 5 seconds and the time interval between injections is 20 seconds. The reference cell is maintained at the same temperature at which the experiment is run by providing constant power to the reference cell. The data obtained from the ITC experiments are analysed using a software from microcal.
2.2 Stopped flow fluorescence

All stopped flow experiments were performed using the Applied Photophysics Ltd (Leatherhead, UK) model SV.17 MV machine with a dead time of 1.7 ms. Stopped flow fluorescence experiments were performed to determine calcium (Ca\(^{2+}\)) binding to CaM in the presence/absence of neurogranin peptides. Two different experiments were performed to study calcium binding.

a) Quin-2 experiments: The Quin-2 experiments were performed as described in (Putkey et al. 2003). CaM and the neurogranin peptides were made up in a buffer whose composition was 20 mM MOPS pH 7.0, 100 mM KCl. The concentration of CaM used was 4 μM in the syringe. Concentration of the peptides to be used was calculated based on the affinity of binding measured from ITC experiments. The calculations are as follows:

From ITC data, we know the stoichiometry of binding of neurogranin and CaM is 1:1. So the binding equation is

\[
\text{Ng} + \text{CaM} \rightleftharpoons \text{Ng-CaM}
\]

\[
K_{\text{on}} [\text{Ng}][\text{CaM}] - K_{\text{off}} [\text{Ng-CaM}]
\]

Grouping similar terms,

\[
(1 + K_{\text{off}}) [\text{Ng-CaM}] = K_{\text{on}} [\text{Ng}][\text{CaM}]
\]

To determine the amount of neurogranin required for 90% binding of neurogranin
and CaM,

\[(1 + K_{off}) \cdot 0.9 \cdot [CaM_0] = K_{on} \cdot [Ng] \cdot (0.1 \cdot [CaM_0])\]

Since \(1 \ll K_{off}\), the equation becomes

\[9K_d = [Ng] \quad (K_d = K_{off} / K_{on})\]

\[[Ng] = [Ng]_0 - [Ng-CaM]\]

\[[Ng]_0 = 9K_d + 0.9 \cdot CaM_0\]

The stopped-flow equipment consists of two injection syringes which inject equal volumes of liquid into a sample cell. The experiment is conducted by injecting equal volumes of liquid from the two syringes which are mixed in the sample cell following which fluorescence is measured. One of the syringes in our experiment contains 150 µM Quin-2 and the other syringe contains CaM and neurogranin peptides. The excitation wavelength was 334.5 nm and the detection of fluorescence emission was controlled by a 435 nm cut-on filter (Oriel #51282). The slit widths were set at 0.5 nm.

b) **Tyrosine fluorescence experiments:** The tyrosine fluorescence experiments were also performed on the stopped-flow equipment. One syringe contained 10 µM CaM, 100 µM CaCl₂ and neurogranin peptides at concentrations calculated as mentioned in the earlier section (to ensure 90% binding under any condition) in 20 mM MOPS pH 7.5, 100 mM KCl and the other syringe contained 5mM EGTA in the same
buffer. The EGTA present in excess absorbs free calcium present. Hence calcium dissociating from CaM does not reassociate and fluorescence change can be measured accurately. Intrinsic tyrosine fluorescence was monitored at an excitation wavelength of 276 nm and emission was measured with a bandpass emission filter with peak transmittance at 355 nm (Oriel #51662).

2.3 Protein expression and purification

a) Neurogranin expression and purification: The cDNAs encoding the full-length RC3 protein was expressed from the pET23 vector in the BL21 DE3 pLys S strain of E. coli. The plasmids were cultured in LB medium with ampicillin and chloramphenicol. When the cultures reached an OD$_{595}$ of 0.4-0.6, protein expression was induced with 200 mg/ml of IPTG and the cultures were maintained at 37°C for 6 hours. The cells were harvested by centrifugation in a JA-10 rotor at a speed of 4000 rpm for 20 minutes at 4°C. To purify RC3, the following steps were performed at 4°C. Cell pellets were lysed in a cold hypotonic lysis buffer (1 mM HEPES, 1 mM DTT, 1 mM PMSF), with sonication. The lysate was centrifuged at 25,000 x g to remove particulate material. The supernatant was brought to 65% ammonium sulfate at 4°C, causing precipitation of RC3. After centrifugation at 10,000 x g for 30 min, the pellet was resuspended in 15 ml of 50 mM Tris, pH 6.8, 50 mM NaCl and perchloric acid was added to 2.5% final concentration. The mixture was centrifuged at 10,000 x g for 15 min, and then the supernatant was brought to 90% ammonium sulfate. After a final spin at 10,000 x g for 30 min, the pellet was resuspended in 10 mM HEPES, 200 mM KCl and dialysed overnight into the same buffer. The next
day, protein concentration was calculated by measuring absorbance at 600 nm and the protein was used in experiments.

b) **CaM expression and purification:** CaM was expressed in BL23 (DE3) star chemically competent cells using IPTG similar to the expression protocol of neurogranin. CaM was purified by resuspending the pellet in 25 ml lysis buffer (50 mM Tris pH 7.5, 10 mM EDTA) following which the pellet was sonicated on ice. The lysate was spun at 39100 x g for 1 hour using a Beckman JA20 rotor. The supernatant was brought to 2.5 M ammonium sulfate at room temperature by addition of ammonium sulfate over a period of 30 minutes to an hour such that it gets into solution slowly. Following centrifugation at 12500 x g for 10 minutes in a JA 20 rotor, the supernatant was brought to saturation with ammonium sulfate. The solution was centrifuged at 12500 x g for 10 minutes in a JA20 rotor and the pellet was resuspended in 50 ml of resuspension buffer (50 mM Tris, pH 7.5, 200 mM ammonium sulfate and 1 mM EDTA). The solution was then loaded over a phenyl sepharose column which was pre-equilibrated with resuspension buffer at room temperature. Calcium chloride was added to 2.5 mM final concentration from a 1M stock to Column I flow through. To the solution was added 200 mM ammonium sulfate slowly over a period of time and this mixture was then added to column II which was pre-equilibrated with wash buffer. To column II, elution buffer was added to elute the fractions and the fraction collected using a fraction collector. The absorbance of each fraction at 276 nm was measured and the fraction with the maximum OD was then dialysed overnight in 50 mM MOPS. The fraction was
concentrated the next day using a Centriplus 3000 MWCO (Millipore cat # 4420) at 4°C.

2.4 MATLAB simulations

The purpose of these MATLAB simulations was to develop a model of interaction between neurogranin, CaM and calcium such that the model can be used to predict interactions under conditions that are difficult to measure experimentally. The model of interaction that I generated here is based on the law of mass action. Under the law of mass action, the rate at which a reaction progresses is dependent on the concentration of the reactants and the reaction rate constants. I wrote out ordinary differential equations (ODE) that described the rate of change of each species of CaM. Some of the kinetic constants used were values reported in literature from experiments. Other kinetic constants which cannot be experimentally determined were fixed by a method called parameter optimization which is described below. Once the constants were determined, the model was calibrated by ensuring that the principle of mass conservation was not violated under any condition. The ODE model was then used to run simulation experiments to predict different results which will be described below.

a) ODE model generation: There are 9 different ‘species’ of CaM in the model based on the calcium saturated state of CaM. These are the apo-CaM, fully calcium-saturated CaM and seven other species of CaM depending on the calcium- saturation state of the N-lobe or C-lobe of CaM. Similarly, there are 9 different ‘species’ of neurogranin bound CaM. There are a total of 66 rate constants that correspond to the rates of association and dissociation of neurogranin, CaM and calcium. Using
general principles that are proven through experiments, it is possible to arrive at certain relationships between the 66 kinetic constants such that the number of constants that need to be determined are 22. Of the 22 constants that need to be determined, 14 were determined using values in literature. There were 8 constants which were determined using the method of parameter optimization that began with a best guess value and arrived at the optimal value after a series of iterations. The parameter optimization process is described in the next section.
Figure 2.1: Pictorial representation of the ODE model with the different species and their reactions

CON0 represents apoCaM, C1N0-CaM with a partially saturated C-lobe, RC0N0-neurogranin bound apoCaM, RC2N2-neurogranin bound to fully saturated CaM.
b) **Parameter Optimization:** Parameter optimization refers to the method that was used to obtain the best possible parameters (on-rates and off-rates) in the ODE model. I used MATLAB simulations to search parameter space for the best possible parameters. The parameter space search was initiated using a seed set of parameters which were the best guess values for parameters.

Using the seed set of parameters, I wrote codes that replicated each of the experiments- ITC and stopped flow fluorimetry- in MATLAB. After each experiment was replicated, I plotted the data obtained in a form similar to what was obtained experimentally. I called this the result of my theoretically conducted experiments. I then plotted the experimental data from ITC and stopped-flow fluorimetry experiments. This was the experimental result. The theoretical result and experimental result were compared and the difference between them calculated. This was called the ‘error margin’. Based on the error margin, the seed set of parameters was updated. The whole process was repeated again with a new set of parameters that minimized the error values. The optimized parameters were obtained when the error margin was minimal and subsequent iterations with new parameter sets did not minimize the error margin.
Table 2.1 Parameters used in ODE model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>k01</td>
<td>On-rate of first calcium binding to C-lobe of CaM</td>
<td>405.6 μM⁻¹ s⁻¹</td>
<td>Lim et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(max)</td>
<td>Rutskoy et al., 2008</td>
</tr>
<tr>
<td>k02</td>
<td>Off-rate of first calcium binding to C-lobe of CaM</td>
<td>5100 s⁻¹</td>
<td>Pushney et al., 2008</td>
</tr>
<tr>
<td>k03</td>
<td>On-rate of second calcium binding to C-lobe of CaM</td>
<td>56.4 μM⁻¹ s⁻¹</td>
<td>Lim et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(max)</td>
<td>Geerens et al., 2004</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Steven et al., 1997</td>
</tr>
<tr>
<td>k04</td>
<td>Off-rate of second calcium binding to C-lobe of CaM</td>
<td>9.1 s⁻¹</td>
<td>Guerber et al., 2004</td>
</tr>
<tr>
<td>k05</td>
<td>On-rate of first calcium binding to N-lobe of CaM</td>
<td>500 μM⁻¹ s⁻¹</td>
<td>Faas et al., 2011</td>
</tr>
<tr>
<td>k06</td>
<td>Off-rate of first calcium binding to N-lobe of CaM</td>
<td>20000 s⁻¹</td>
<td>Faas et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(max)</td>
<td>Lim et al., 1990</td>
</tr>
<tr>
<td>k07</td>
<td>On-rate of second calcium binding to N-lobe of CaM</td>
<td>12000 μM⁻¹ s⁻¹</td>
<td>Lim et al., 1990</td>
</tr>
<tr>
<td>k08</td>
<td>Off-rate of second calcium binding to N-lobe of CaM</td>
<td>3000 s⁻¹</td>
<td>Faas et al., 2011</td>
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<tr>
<td>k11</td>
<td>On-rate of first calcium binding to C-lobe of R3boundCaM</td>
<td>56.6 μM⁻¹ s⁻¹</td>
<td>Parameter optimization using an ODE model of interaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k12</td>
<td>Off-rate of first calcium binding to C-lobe of R3boundCaM</td>
<td>15657 s⁻¹</td>
<td>Parameter optimization using an ODE model of interaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k13</td>
<td>On-rate of second calcium binding to C-lobe of R3boundCaM</td>
<td>484 μM⁻¹ s⁻¹</td>
<td>Parameter optimization using an ODE model of interaction</td>
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<td></td>
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<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k14</td>
<td>Off-rate of second calcium binding to C-lobe of R3boundCaM</td>
<td>29.6 s⁻¹</td>
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<td></td>
<td></td>
<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k15</td>
<td>On-rate of first calcium binding to N-lobe of R3boundCaM</td>
<td>500 μM⁻¹ s⁻¹</td>
<td>Based on assumption that R3 does not change kinetics of calcium association to N-lobe k15=405</td>
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<td></td>
<td></td>
<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k16</td>
<td>Off-rate of first calcium binding to N-lobe of R3boundCaM</td>
<td>20000 s⁻¹</td>
<td>Based on assumption that R3 does not change kinetics of calcium association to N-lobe k16=405</td>
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<td></td>
<td></td>
<td>(rated value)</td>
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<td>k17</td>
<td>On-rate of second calcium binding to N-lobe of R3boundCaM</td>
<td>1200 μM⁻¹ s⁻¹</td>
<td>Based on assumption that R3 does not change kinetics of calcium association to N-lobe k17=407</td>
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<td></td>
<td></td>
<td>(rated value)</td>
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<tr>
<td>k18</td>
<td>Off-rate of second calcium binding to N-lobe of R3boundCaM</td>
<td>8000 s⁻¹</td>
<td>Based on assumption that R3 does not change kinetics of calcium association to N-lobe k18=408</td>
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<td></td>
<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k101</td>
<td>On-rate of R3 binding to apo-CaM</td>
<td>60.6 μM⁻¹ s⁻¹</td>
<td>Calculated using Kd values obtained from ITC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k102</td>
<td>Off-rate of R3 binding to apo-CaM</td>
<td>38.7 s⁻¹</td>
<td>Parameter optimization using an ODE model of interaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k103</td>
<td>On-rate of R3 binding to partially saturated CaM</td>
<td>90 μM⁻¹ s⁻¹</td>
<td>Calculated from the Kd values obtained from ITC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k104</td>
<td>Off-rate of R3 binding to partially saturated CaM</td>
<td>1671 s⁻¹</td>
<td>Calculated using the principle of microscopic reversibility</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k105</td>
<td>On-rate of R3 binding to saturated CaM</td>
<td>75 μM⁻¹ s⁻¹</td>
<td>Parameter optimization using an ODE model of interaction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(rated value)</td>
<td></td>
</tr>
<tr>
<td>k106</td>
<td>Off-rate of R3 binding to saturated CaM</td>
<td>225 s⁻¹</td>
<td>Calculated using the principle of microscopic reversibility</td>
</tr>
</tbody>
</table>

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c) **Simulation experiments:** Once the ODE model was calibrated and the parameters determined, I ran simulations to test different phenomena. When there is a calcium influx, the N-lobe of CaM saturates first followed by a redistribution of calcium to the C-lobe of CaM. To test the effect of neurogranin on CaM’s mode of calcium binding, I studied the time-dependent change in concentrations of N-lobe saturated CaM, C-lobe saturated CaM and fully saturated CaM following a calcium influx.

One of the questions of great significance in studies on CaM is the effect that proteins binding to CaM have on CaM’s calcium binding properties. To address this question, I performed a simulation experiment where I introduced calcium (20 µM) into an equilibrated system of CaM (20 µM) and neurogranin (20 µM). Following the injection of calcium into the system, I followed the concentrations of calcium-saturated CaM, apo-CaM, apo-CaM bound to neurogranin and calcium-saturated CaM bound to neurogranin to study the effect of neurogranin on calcium saturation of CaM. The results of the experiment will be discussed and interpreted in the discussion section. In the next experiment, the steady-state concentrations of calcium-saturated CaM and neurogranin bound calcium-saturated CaM was determined at different concentrations of neurogranin. This is a direct test of the effect of neurogranin on calcium saturation of CaM.
CHAPTER 3

RESULTS AND

DISCUSSION
3.1 Overview

CaM plays a pivotal role in synaptic plasticity since this protein is activated by binding to calcium following which it activates other molecules downstream, many of which affect plasticity at the synapse by a variety of biochemical and biophysical means. As the primary calcium signal transducer, CaM serves to transduce calcium signals that are intense, transient and diverse. CaM acts a versatile calcium sensor that responds to all these diverse calcium signals by virtue of having four calcium binding sites each of which have their own kinetic and equilibrium rates of calcium binding and dissociation. Furthermore, various proteins like small neuronal IQ domain proteins that interact with CaM alter the kinetics of calcium binding of some of these sites providing versatility to CaM.

The protein of focus is neurogranin, a small neuronal IQ domain protein abundant in the neurons of hippocampus and frontal lobe cortex. Research into the interaction of neurogranin with CaM suggested that neurogranin accelerated the rate of calcium dissociation from CaM (Gaertner, Putkey, and Waxham 2004). To establish unequivocally the role of neurogranin in regulating CaM, it is necessary to have quantitative data on neurogranin’s interaction with CaM as well as its effect on the kinetics and dynamics of calcium binding to CaM. I used Isothermal Titration Calorimetry (ITC) to study the thermodynamics of CaM association with neurogranin and stopped-flow fluorimetry to study the kinetics of calcium binding to CaM in the presence of neurogranin peptides. In all my experiments, I used peptides that represent different regions of the protein and in some cases a phosphorylated state of the protein.
3.2 Thermodynamics of CaM association with neurogranin

Thermodynamic analysis of the interaction of CaM with other molecules provides useful information on the regulation of CaM by a variety of these molecules. Studies on troponin I-CaM interaction as well as myosin light chain kinase-CaM interaction by Daniel Storm and colleagues demonstrated that the free energy coupling for each CaM-protein complex is unique and that protein interaction with CaM can change the free energy coupling for calcium binding to CaM (Olwin et al. 1984; Keller et al. 1982). To determine the energetics of calcium binding to CaM and the influence of neurogranin peptides, I used isothermal titration calorimetry (ITC) and measured the exothermic change that occurred when CaM interacted with neurogranin peptides or the protein. Some of the experiments were conducted in a buffer containing 50 mM HEPES in the presence of 150 mM KCl to mimic physiological ionic concentrations.

In this section, I will focus on the dissociation constant values obtained from the ITC measurements and aim to interpret them in the context of the underlying biology. Dissociation constant, $K_d$, is a specific type of equilibrium constant that is used as a measure of the propensity of a complex to dissociate into its component molecules. $K_d$ is also defined as the ratio of the off-rate constant and on-rate constant:

$$K_d = \frac{k_{off}}{k_{on}}$$

where $k_{off}$ is the off-rate constant that is the rate of the reverse reaction and $k_{on}$ is the on-rate constant that refers to the rate of the forward reaction. When a reaction between molecules has a high $K_d$, it means that either the off-rate constant has a high value or the on-rate constant has a low value or it is a combination of both. Hence, there is an inverse
relationship between $K_d$ and affinity and an increase in $K_d$ indicates reduced affinity and vice versa.

### 3.2.1 RC3 (26-49) interaction with CaM

I began thermodynamics study on the neurogranin peptides with RC3 (26-49) peptide. The RC3 (26-49) peptide contains only the IQ domain region of the peptide with a few amino acids N-terminal to it. NMR and CD spectroscopy studies on the neurogranin protein have established that this region of the protein forms an $\alpha$-helix upon binding to apo-CaM in the presence of salt (Gerendasy et al. 1995; Ran et al. 2003). Furthermore, chemical shifts of the residues in the neurogranin protein show that many residues in the IQ region of the protein show significant structural changes upon binding to CaM (Ran et al. 2003). NMR data along with stopped-flow fluorimetry data on PEP-19 peptides also indicated that the IQ domain region of the peptide provides a targeting function that allows binding to CaM (Putkey et al. 2008). Since a key role has been attributed to the IQ domain of neurogranin in binding to CaM, I wanted to determine the binding affinity of the IQ domain region of neurogranin to CaM.
Figure 3.1: Result of RC3 (26-49) peptide interaction with CaM

**RC3 (26-49) PEPTIDE**

Experimental condition: No salt

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3 (26-49) peptide with apoCaM</td>
<td>N= 1.35</td>
</tr>
<tr>
<td></td>
<td>$K_d=630.2 \text{ nM}$</td>
</tr>
<tr>
<td>RC3(26-49) peptide with $Ca^{2+}$/CaM</td>
<td>N= 1.04</td>
</tr>
<tr>
<td></td>
<td>$K_d=874.33 \text{ nM}$</td>
</tr>
</tbody>
</table>
**Discussion of RC3 (26-49) result:**

The RC3 (26-49) peptide bound with high affinities of 0.63 µM to apo-CaM and 0.87 µM to calcium-CaM in the absence of salt. Interestingly, binding in the presence of salt was below detection. Crystal structure studies by Quiocio and colleagues on skMLCK and CaMKII binding to CaM showed that CaM formed a compact structure with a hydrophobic tunnel that runs at an angle of 45 degrees to the long axis of the protein (Meador, Means, and Quiocio 1992; Meador, Means, and Quiocio 1993). The target peptide in both the above cases formed an α-helix whose hydrophobic residues interacted with the hydrophobic tunnel. The length of the α-helix dictated the degree of compactness of CaM. It is possible that RC3 (26-49), a short peptide, which forms an α-helix upon interacting with CaM, does not cause CaM to form a highly compact structure and hence there are fewer points of contact between the peptide and the protein. The crystal structure of RC3(27-50) peptide interaction with CaM predicts that there are three residues- Ile 33, Ser 36 and Arg 38 that mainly interact with Cam residues. Presence of salt might be able to easily disrupt the interactions.

**3.2.2 RC3 (13-49) interaction with CaM**

The acidic domain N-terminal to the IQ domain has been implicated in binding as well. Hence I next studied the RC3 (13-49) peptide which has both the IQ domain region and the acidic region N-terminal to it. Represented below in figures are the ITC data for binding between RC3 (13-49) peptide and CaM under different conditions (absence/presence of saturating concentrations of calcium, 150 mM KCl).
Figure 3.2: Result of RC3 (13-49) peptide interaction with CaM- No salt present

RC3 (13-49) PEPTIDE

Experimental condition: No salt

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3 (13-49) peptide with apoCaM</td>
<td>N = 0.92</td>
</tr>
<tr>
<td></td>
<td>$K_d = 66.2$ nM</td>
</tr>
<tr>
<td>RC3 (13-49) peptide with $\text{Ca}^{2+}$/CaM</td>
<td>N = 1.07</td>
</tr>
<tr>
<td></td>
<td>$K_d = 1.25$ $\mu$M</td>
</tr>
</tbody>
</table>
Figure 3.3: Result of RC3 (13-49) peptide interaction with CaM- Salt present

**RC3(13-49) PEPTIDE**

Experimental condition: Salt Present

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3 (13-49) peptide with apoCaM</td>
<td>N - 1.08</td>
</tr>
<tr>
<td></td>
<td>$K_d - 639.3\ nm$</td>
</tr>
<tr>
<td>RC3 (13-49) peptide with Ca^{2+}/CaM</td>
<td>N - 1.07</td>
</tr>
<tr>
<td></td>
<td>$K_d - 3.01\ \mu M$</td>
</tr>
</tbody>
</table>
**Discussion of RC3 (13-49) results:**

In the absence of salt, RC3 (13-49) peptide bound with a high affinity $K_d$ of 0.066 µM to apo-CaM. Binding to calcium/CaM was much weaker with a $K_d$ of 1.25 µM that is 18 times lower. In the presence of physiological concentrations of salt (150 mM KCl), the affinity of binding to apo-CaM weakened ten-fold with a $K_d$ of 0.64 µM. The affinity of calcium/CaM to neurogranin was 2.4 fold lower in the presence of salt with a $K_d$ of 3.01 µM. (The number of trials for each peptide for each condition was 2).

Earlier work on the PEP-19 peptides and the protein indicated that the IQ domain provided a targeting function that allowed binding of PEP-19 to CaM whereas the acidic domain modified the nature of this interaction and was essential for modulating calcium binding to the C-domain of CaM(Putkey et al. 2008). Thus both the IQ domain and the acidic domain N-terminal to it were deemed necessary for binding of PEP-19 and CaM. The RC3 (13-49) results suggest that the regions of the neurogranin protein behave in a similar fashion to the PEP-19 protein.

**3.2.3 RC3 Protein**

In order to determine with certainty that the RC3 (13-49) peptide behaved in a very similar fashion to the RC3 protein and also to study the properties of the protein itself, I measured the enthalpy, entropy and binding affinity of the RC3 protein.
Figure 3.4: Result of RC3 protein interaction with CaM

RC3 PROTEIN

Experimental condition: No salt

a) apoCaM binding

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3 protein with apoCaM</td>
<td>$K_{d}=71$ nM</td>
</tr>
<tr>
<td>RC3 protein with Ca$^{2+}$/CaM</td>
<td>$K_{d}=1.39$ $\mu$M</td>
</tr>
</tbody>
</table>
Discussion of RC3 protein results

The neurogranin protein bound to apo-CaM with an affinity of 0.071 µM similar to that of the RC3 (13-49) peptide (K_d of 0.066 µM). K_d for interaction between calcium-CaM and neurogranin protein was 1.39 µM similar again to RC3 (13-49) peptide which had a K_d of 1.25 µM. These interactions were in the absence of salt. However, in the presence of salt, binding between the neurogranin protein and CaM was below detection.

3.2.4 RC3 (13-52) peptide interaction with CaM

NMR characterization of the neurogranin-CaM interaction led to the identification of residues in neurogranin that showed significant chemical shifts and hence are hypothesized as regions of interaction between CaM and neurogranin(Ran et al. 2003). Three acidic residues E, C and G which are C-terminal to the IQ motif were found to show significant chemical shifts and hence it is believed that these three residues are important for neurogranin-CaM interaction. To test the contribution of these residues in binding, we created a peptide with the three residues added to the C-terminal of the RC3 (13-49) peptide. I measured the binding affinity between this peptide and CaM using ITC measurements.
Figure 3.5: Result of RC3 (13-52) peptide interaction with CaM-No salt

**RC3 (13-52) PEPTIDE**

**Experimental condition: No salt**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3 (13-52) peptide with apoCaM</td>
<td>N = 1.35</td>
</tr>
<tr>
<td></td>
<td>$K_d = 158.5$ nM</td>
</tr>
<tr>
<td>RC3(13-52) peptide with Ca$^{2+}$/CaM</td>
<td>N = 1.25</td>
</tr>
<tr>
<td></td>
<td>$K_d = 2.36$ µM</td>
</tr>
</tbody>
</table>
Figure 3.6: Result of RC3 (13-52) peptide interaction with CaM- salt present

**RC3 (13-52) PEPTIDE**

Experimental condition: Salt present

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Result</th>
</tr>
</thead>
</table>
| RC3 (13-52) peptide with apoCaM | $N$- 1.48  
$K_e$ 909.2 nM |
| RC3 (13-52) peptide with Ca$^{2+}$/CaM | $N$- 1.29  
$K_d$ 4.61 μM |
Discussion of RC3 (13-52) peptide results

In a demonstration that greater number of points of contact need not correspond to increased affinity, the $K_d$ between the RC3 (13-52) peptide and apo-CaM was found to be 0.158 µM which is 2.5 times higher than the $K_d$ between RC3 (13-49) peptide and apo-CaM in the absence salt. Similarly, $K_d$ of binding of RC3 (13-52) to calcium-CaM was 2.36 µM compared to 1.25 µM for RC3 (13-49) peptide. The dissociation constants were 0.91 µM and 4.61 µM for interaction between RC3 (13-52) peptide and apo-CaM and calcium-CaM respectively in the presence of 150 mM KCl. These values are only modest increases of 1.4 fold from the 0.64 µM measured with RC3 (13-49) for apo-CaM interaction and 1.5 fold from the value of 3.01 µM for calcium-CaM interaction with RC3 (13-49).

3.2.5 RC3 (S36D) peptide with CaM

Phosphorylation of neurogranin by protein kinase C is a feedback mechanism that regulates activity of neurogranin by potentially changing its affinity to CaM. Observations made by Ramakers and group on the phosphorylated state of neurogranin following induction of long-term potentiation and long-term depression strengthened the idea of phosphorylation as a feedback mechanism for neurogranin regulation (Ramakers et al. 1995).

The S36D variant of neurogranin has been used as a phospho-mimetic in literature (Gerendasy et al. 1994) where competition experiments showed that this variant bound with lower affinity to CaM than the wildtype protein. I determined the binding affinity of this variant with CaM in the presence and absence of salt.
Figure 3.7: Result of RC3 S36D peptide interaction with CaM- No salt

RC3 S36D PEPTIDE

Experimental condition: No salt

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3 S36D with apoCaM</td>
<td>N=1.09</td>
</tr>
<tr>
<td></td>
<td>$K_a = 2.29$ uM</td>
</tr>
<tr>
<td>RC3 S36D with Ca$^{2+}$/CaM</td>
<td>N=0.95</td>
</tr>
<tr>
<td></td>
<td>$K_e = 5.27$ uM</td>
</tr>
</tbody>
</table>
Discussion of RC3 (S36D) peptide results

In the absence of salt, RC3 S36D peptide bound to apo-CaM with a $K_d$ of 2.29 uM and to calcium-CaM with a $K_d$ of 5.27 uM. In the presence of salt, binding was below detection. Calcium binding experiments with calbindin D$_{9k}$ demonstrated that salt serves to shield the charges on the surface of calbindin D$_{9k}$ (Linse, Helmersson, and Forsén 1991; Linse et al. 1988). It is possible that the presence of salt shields the negative charge of RC3 S36D and reduces binding affinity to below detectable levels.

3.2.6 RC3 (13-49) peptide with CaM at high temperature

I next looked at the behavior of the RC3 (13-49) peptide at higher temperatures (37°C) to study the effect of temperature on binding affinities.
Figure 3.8: Result of RC3 (13-49) peptide interaction with CaM at 37°C - No salt

RC3 (13-49) PEPTIDE- 37C

Experimental condition: No salt

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3(13-49) with apoCaM</td>
<td>N= 1.25</td>
</tr>
<tr>
<td></td>
<td>K_d= 0.43 μM</td>
</tr>
<tr>
<td>RC3(13-49) with Ca^{2+} CaM</td>
<td>N= 1.305</td>
</tr>
<tr>
<td></td>
<td>K_d=1.86 uM</td>
</tr>
</tbody>
</table>
Figure 3.9: Result of RC3 (13-49) peptide interaction with CaM at 37°C- Salt present

**RC3(13-49) PEPTIDE- 37°C**

Experimental condition: Salt Present

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3 (13-49) with apo CaM</td>
<td>N- 1.35</td>
</tr>
<tr>
<td></td>
<td>K_a- 2.64 μM</td>
</tr>
<tr>
<td>RC3 (13-49) with Ca^{2+}/CaM</td>
<td>N- 1.095</td>
</tr>
<tr>
<td></td>
<td>K_a- 4.59 μM</td>
</tr>
</tbody>
</table>
Discussion of RC3 (13-49) at high temperature

The dissociation constant between apo-CaM and RC3 (13-49) peptide at 37°C in the absence of salt was 0.43 µM whereas at 25°C it was 0.066 µM. Dissociation constant between apo-CaM and RC3(13-49) peptide in the presence of 150 mM KCl at 37°C was 2.64 µM and at 25°C it was 0.639 µM. In contrast, dissociation constant between RC3(13-49) peptide and calcium-CaM was not very different being 1.86 µM in the absence of salt at 37°C and 1.25 µM at 25°C. The dissociation constant between calcium-CaM and RC3 (13-49) in the presence of salt at 25°C was 3.01 µM and at 37°C it was 4.59 µM.

A common theme running through all the binding data from ITC experiments is that salt lowers the binding affinity between neurogranin peptides and CaM. There is precedent for this effect in literature. The effect of salt on affinity of binding between CaM and P-57 (neuromodulin) was examined by Daniel Storm and colleagues (Alexander et al. 1987). They determined that the dissociation constant ($K_d$) between apo-CaM and P-57 increased from 0.23 µM in the absence of salt to 3.4 µM in the presence of 150 mM KCl. The dissociation constant ($K_d$) between calcium-CaM and P-57 increased from 1 µM in the absence of salt to 3 µM in the presence of 150 mM KCl.
Table 3.1: Summary of affinity of interaction between CaM and neurogranin protein/peptides

<table>
<thead>
<tr>
<th>Protein/Peptide used</th>
<th>Interaction with apo-CaM</th>
<th>Interaction with calcium-CaM</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3(26-49) peptide</td>
<td>N -1.55</td>
<td>K_d = 630.2 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K_d = 874.33 nM</td>
</tr>
<tr>
<td>RC3(13-49) peptide</td>
<td>N -0.92</td>
<td>K_d = 66.2 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K_d = 1.25 μM</td>
</tr>
<tr>
<td>RC3(13-52) peptide</td>
<td>N -1.35</td>
<td>K_d = 158.5 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K_d = 2.96 μM</td>
</tr>
<tr>
<td>RC3(13-49) peptide at 37°C</td>
<td>N -1.25</td>
<td>K_d = 1.86 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K_d = 1.86 μM</td>
</tr>
<tr>
<td>RC3(536D) peptide</td>
<td>N -1.09</td>
<td>K_d = 2.29 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>K_d = 5.27 μM</td>
</tr>
<tr>
<td>RC3 protein</td>
<td>K_d = 71 nM</td>
<td>K_d = 1.99 μM</td>
</tr>
</tbody>
</table>

Experimental condition: No salt

3.3 Enthalpic and entropic changes associated with CaM-neurogranin interaction

Enthalpy is a measure of the total energy needed by a thermodynamic system. It is used as a measure of the changes in physical, chemical and biological measurements in a system. Entropy is yet another mathematically defined function that helps to account for the heat flow through a thermodynamic system. The statistical mechanics interpretation of entropy is that it is a measure of randomness or disorder in a system. However, it is also
possible to use entropy to study protein-protein interaction. When proteins interact, hydrophobic surfaces are buried and hydrophilic surfaces are exposed to the solvent. There is release of water when hydrophobic surfaces get buried within the protein also known as ‘energy of hydration’. There is an entropic change associated with this energy of hydration called solvent entropy. There are changes to rotational freedom of individual residues within each protein as proteins interact and these changes are encapsulated as conformational entropy. There is also another entropy term called vibrational entropy which is the change in vibrational flexibility of protein when secondary structures are stabilized (Schuler, Lipman, and Eaton 2002; Cho et al. 1996).

Linse and colleagues devised an effective way to use thermodynamic measurements to study the effect of individual charged residues on calcium binding to the protein calbindin D$_{9K}$ (Linse et al. 1988). This group measured macroscopic binding constants by titrating different mutants of calbindin D$_{9K}$ (mutations were made to neutralize the different charged residues) with calcium in the presence of calcium chromophores BAPTA or Quin-2 and measuring the change in absorbance at 263 nm. From the macroscopic binding constants, free energy change was calculated and the effect of each charged residue on free energy change and its biological implication was predicted.

I explained the concepts of entropy and enthalpy as well as ways in which measurements on biological systems are used to determine thermodynamic constants that are then used to interpret the underlying biology because I will be performing similar calculations to calculate free energy and attempt to interpret the data in a similar fashion.
In my calorimetry experiments, I used a single mutant peptide RC3 S36D and other peptides to determine change in entropy and enthalpy. Using these thermodynamic measurements, I calculated $\Delta G$, Gibbs free energy as follows. At constant temperature,

$$\Delta G = \Delta H - T\Delta S$$

**Table 3.2: Enthalpic and entropic changes following RC3 peptide interaction with CaM**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Experimental condition</th>
<th>Enthalpy change ($\Delta H$)</th>
<th>Entropy change ($\Delta S$)</th>
<th>Free energy change ($\Delta G$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3 (26-49) peptide with apo-calmodulin</td>
<td>No salt</td>
<td>8298.00</td>
<td>0.57</td>
<td>8118.14</td>
</tr>
<tr>
<td>RC3 (26-49) peptide with calcium-calmodulin</td>
<td>No salt</td>
<td>2817.33</td>
<td>18.33</td>
<td>-2645.01</td>
</tr>
<tr>
<td>RC3 (13-49) peptide with apo-calmodulin</td>
<td>No salt</td>
<td>-1.68E4</td>
<td>-23.40</td>
<td>-9126.00</td>
</tr>
<tr>
<td>RC3 (13-49) peptide with calcium-calmodulin</td>
<td>No salt</td>
<td>-4629.50</td>
<td>11.45</td>
<td>-5041.60</td>
</tr>
<tr>
<td>RC3 (13-52) peptide with apo-calmodulin</td>
<td>No salt</td>
<td>-1.49E4</td>
<td>-18.65</td>
<td>-9342.30</td>
</tr>
<tr>
<td>RC3 (13-52) peptide with calcium-calmodulin</td>
<td>No salt</td>
<td>-4004.50</td>
<td>12.35</td>
<td>-7684.80</td>
</tr>
<tr>
<td>RC3 S36D peptide with apo-calmodulin</td>
<td>No salt</td>
<td>-5252.00</td>
<td>8.38</td>
<td>-7749.49</td>
</tr>
<tr>
<td>RC3 S36D peptide with calcium-calmodulin</td>
<td>No salt</td>
<td>-1535.66</td>
<td>19.033</td>
<td>-7267.49</td>
</tr>
<tr>
<td>RC3 protein with apo-calmodulin</td>
<td>No salt</td>
<td>-9646.00</td>
<td>0.051</td>
<td>-9630.80</td>
</tr>
<tr>
<td>RC3 protein with calcium-calmodulin</td>
<td>No salt</td>
<td>-6616.00</td>
<td>3.55</td>
<td>-7673.90</td>
</tr>
<tr>
<td>RC3 (26-49) peptide with apo-calmodulin</td>
<td>Salt present (150 mM KCl)</td>
<td>Below detection</td>
<td>Below detection</td>
<td>Below detection</td>
</tr>
<tr>
<td>RC3 (26-49) peptide with calcium-calmodulin</td>
<td>Salt present (150 mM KCl)</td>
<td>Below detection</td>
<td>Below detection</td>
<td>Below detection</td>
</tr>
<tr>
<td>RC3 (13-49) peptide with apo-calmodulin</td>
<td>Salt present (150 mM KCl)</td>
<td>-7317.00</td>
<td>3.80</td>
<td>-8149.40</td>
</tr>
<tr>
<td>RC3 (13-49) peptide with calcium-calmodulin</td>
<td>Salt present (150 mM KCl)</td>
<td>-3463.67</td>
<td>13.03</td>
<td>-7585.01</td>
</tr>
<tr>
<td>RC3 (13-52) peptide with apo-calmodulin</td>
<td>Salt present (150 mM KCl)</td>
<td>-4905.50</td>
<td>11.62</td>
<td>-8368.26</td>
</tr>
<tr>
<td>RC3 (13-52) peptide with calcium-calmodulin</td>
<td>Salt present (150 mM KCl)</td>
<td>-1972.00</td>
<td>17.8</td>
<td>-7276.40</td>
</tr>
<tr>
<td>RC3 S36D peptide with apo-calmodulin</td>
<td>Salt present (150 mM KCl)</td>
<td>Below detection</td>
<td>Below detection</td>
<td>Below detection</td>
</tr>
<tr>
<td>RC3 S36D peptide with calcium-calmodulin</td>
<td>Salt present (150 mM KCl)</td>
<td>Below detection</td>
<td>Below detection</td>
<td>Below detection</td>
</tr>
<tr>
<td>RC3 protein with apo-calmodulin</td>
<td>Salt present (150 mM KCl)</td>
<td>Low signal to noise</td>
<td>Low signal to noise</td>
<td>Low signal to noise</td>
</tr>
<tr>
<td>RC3 protein with calcium-calmodulin</td>
<td>Salt present (150 mM KCl)</td>
<td>Low signal to noise</td>
<td>Low signal to noise</td>
<td>Low signal to noise</td>
</tr>
</tbody>
</table>

Looking across the table of enthalpy, entropy values there are no immediate conclusions that can be drawn based solely on the values as there seems to be no pattern that emerges within the values. However, calculation of free energy for each interaction provides insight into the nature of interaction between RC3 peptides and CaM and calcium.

For instance, the free energy of interaction of calcium to RC3 (26-49) peptide and CaM can be calculated by subtracting the free energy value obtained for RC3 (26-49)
interaction with apo-CaM from the value obtained for RC3 (26-49) interaction with calcium-CaM. Similar calculations can be made for each of the other peptide-CaM interactions. The results show that the free energy for calcium interaction with RC3 (26-49) peptide-CaM complex is -10763.15 kcal in comparison to 1785.2 kcal for calcium interaction with RC3 (13-49) peptide-CaM complex. The biological significance of this finding is that the affinity of calcium is decreased 6-fold when RC3 (13-49) peptide is in complex with CaM.

RC3 S36D has a free energy change of 541.75 kcal for interaction with calcium. Comparing it to the value obtained for RC3 (13-49) peptide-1785.2 kcal, it shows that mutation of a single residue in RC3 (13-49) resulted in a 3.3 fold reduction in calcium affinity. Finally, based on free energies of calcium binding, the RC3 (13-49) peptide is the peptide that best mimics RC3 protein.

3.4 Kinetics of calcium binding

The rate of calcium binding is a constant of interest since biological systems are dynamic systems where changes could occur within a few milliseconds. To study the kinetics of calcium binding to CaM, I used two different readouts. In one set of experiments, I studied fluorescence changes in the tyrosine residues intrinsically present in the C-terminal lobe of CaM. CaM has two tyrosine residues in the C-terminal lobe. Tyrosine is excited at 276 nm and peak emission at 304 nm is monitored. When calcium binds to the C-terminal lobe of CaM there is a change in tyrosine fluorescence. This change in fluorescence is plotted against the free calcium concentration to obtain the $K_d$ of calcium binding. The other readout I used is the fluorescence of a calcium chromophore, Quin-2. The excitation wavelength is 334.5 nm and the emission at 435 nm is measured. Quin-2 fluorescence
increases upon binding calcium. The advantage in using the Quin-2 system is that it can measure all calcium released from CaM as opposed to tyrosine fluorescence which measures only the calcium released from the C-terminal of CaM.

In the first set of experiments, I measured increase in Quin-2 fluorescence as calcium dissociated from CaM. Quin-2 is a calcium chelator with a high affinity towards calcium ($K_d$ of 74 nM) and an extremely fast on-rate of calcium binding-$10^9$ M$^{-1}$ S$^{-1}$ (Lakowicz et al. 1992; Bayley et al. 1984) which makes it a suitable calcium chelator to measure calcium dissociation from CaM.
3.4.1 Quin-2 fluorescence of calcium dissociation from CaM

Figure 3.10: Fluorescence due to calcium release from CaM measured using Quin-2, a calcium dye

Discussion of calcium dissociation from CaM measured from Quin-2 fluorescence

CaM only - 12.93 s\(^{-1}\)

Calcium dissociation rate (k\(_{off}\)) from CaM was at the rate of 12.93 s\(^{-1}\) at 22°C. Stopped flow experiments by Putkey and group under similar experimental conditions yielded a dissociation rate of 9.9±0.4 s\(^{-1}\) for calcium dissociation from CaM(Putkey et al. 2008). A value of 9.1±0.6 s\(^{-1}\) was reported for calcium dissociation from CaM under identical conditions in (Gaertner, Putkey, and Waxham 2004). Values for calcium dissociation from the C-domain of CaM using stopped flow were reported between 8.5 s\(^{-1}\)
and 12.6 s⁻¹ (Peersen, Madsen, and Falke 1997; Persechini, White, and Gansz 1996). The value of 12.93 s⁻¹ that I report here aligns well with the values reported in literature.

### 3.4.2 Quin-2 fluorescence for RC3 (13-49) peptide complex with CaM

Following the measurement with CaM, I studied the effect of the RC3 (13-49) peptide on the calcium dissociation rate from CaM. Kinetic studies on peptides of PEP-19, another SNIQ domain protein family member indicated that the peptide containing the IQ domain along with a few residues N-terminal to the IQ domain accelerated calcium release from CaM to 390±40 s⁻¹ from 9.9±0.4 s⁻¹ (Putkey et al. 2008). Since the IQ domain of PEP-19 and neurogranin share sequence homology, it was an interesting question to see if the neurogranin long peptide behaved in an analogous manner to PEP-19 (28-62).
Figure 3.11: Fluorescence due to calcium release from CaM in the presence of RC3 (13-49) peptide measured using Quin-2, a calcium dye

Discussion of quin-2 fluorescence with RC3 (13-49) peptide interaction with CaM

The neurogranin long peptide accelerated calcium dissociation to 447.25 s⁻¹, a 35-fold increase in $k_{off}$ rate from that of CaM (12.93 s⁻¹) just like the 39-fold increase in $k_{off}$ rate from 9.9±0.4 s⁻¹ to 390±40 s⁻¹ caused by PEP-19 (28-62). This suggests that the domains of
neurogranin interact with CaM to cause functional changes in a manner similar to the domains of PEP-19.

3.4.3 Quin-2 fluorescence of RC3 (26-49) peptide interaction with CaM

I next studied the effect of the IQ domain of neurogranin on calcium dissociation from CaM. Earlier work on the IQ domain of PEP-19 indicated that the IQ domain of PEP-19 reduced the dissociation rate by a factor of 3 from $9.9\pm0.4\ \text{s}^{-1}$ to $3.2\pm0.3\ \text{s}^{-1}$ (Putkey et al. 2008).
Discussion of quin-2 fluorescence RC3 (26-49) peptide in complex with CaM

The IQ domain of neurogranin also reduced the dissociation rate of calcium from CaM from 12.93 s\(^{-1}\) to 2.032 s\(^{-1}\) (6 times lower) similar to the IQ domain of PEP-19 which reduces the rates of calcium dissociation.
3.4.4 Quin-2 fluorescence of RC3 (13-52) peptide interaction with CaM

Structural and dynamic characterization of neurogranin interaction with CaM using NMR revealed the involvement of residues glutamate, cysteine and glycine at the C-terminal end of neurogranin in binding CaM (Ran et al. 2003). I looked at the effect of the peptide containing residues D13 to G52 on calcium dissociation from CaM.

Figure 3.13: Fluorescence due to calcium release from CaM in the presence of RC3 (13-52) peptide measured using Quin-2, a calcium dye
Discussion of Quin-2 fluorescence of RC3 (13-52) peptide interaction with CaM

The RC3 (13-52) peptide unlike the RC3 (13-49) peptide caused two rates of calcium dissociation. One of the rates is the same as calcium dissociation from the C-terminal lobe of CaM. At first pass it would seem that the RC3(13-52) peptide does not change calcium dissociation rate from the C-terminal lobe but on comparison with tyrosine fluorescence it would appear that the RC3 (13-52) peptide affects the kinetics of calcium dissociation in a complex way.

3.4.5 Tyrosine fluorescence of CaM

I looked at intrinsic tyrosine fluorescence from the C-terminal lobe of CaM. CaM has two tyrosine residues in its C-terminal lobe which can be excited at 280 nm. The fluorescence at 304 nm is measured which determines the calcium bound state of the C-terminal lobe of CaM. Tyrosine fluorescence determined in tandem with Quin-2 fluorescence helps delineate the effect of neurogranin peptides on calcium dissociation from the C-terminal lobe and N-terminal lobe.
Discussion of tyrosine fluorescence of CaM

Tyrosine fluorescence measurements of CaM resulted in a rate of 11.67 s\(^{-1}\). This falls well within the range of values reported in literature for calcium dissociation from the C-terminal lobe of CaM ((Peersen, Madsen, and Falke 1997; Persechini, White, and Gansz 1996)).
3.4.6 Tyrosine fluorescence of RC3 (13-49) peptide with CaM

I next looked at tyrosine fluorescence of CaM in the presence of RC3 (13-49) peptide. By comparing the quin-2 fluorescence and tyrosine fluorescence results it is possible to delineate the effect of each of the peptides on calcium binding and dissociation from C-terminal lobe and the N-terminal lobe of CaM.

Figure 3.15: Fluorescence due to calcium release from CaM in the presence of RC3 (13-49) peptide measured using intrinsic tyrosine fluorescence
Discussion of RC3 (13-49) peptide interaction with CaM

The tyrosine fluorescence results show that the RC3 (13-49) peptide accelerates calcium dissociation from the C-terminal of CaM to 285.79 s$^{-1}$. Comparing this result with the quin-2 fluorescence, it is clear that the calcium that dissociated from CaM and was reported by quin-2 was not from the C-lobe alone. The calcium coming off from the N-lobe was also partially reported by quin-2 fluorescence.

3.4.7 Tyrosine fluorescence of RC3 (26-49) peptide with CaM

Figure 3.16: Fluorescence due to calcium release from CaM in the presence of RC3 (26-49) peptide measured using intrinsic tyrosine fluorescence
Discussion of RC3 (26-49) peptide interaction with CaM

The RC3 (26-49) peptide decelerated the rate of calcium dissociation to 2.83 s\(^{-1}\). Comparing this result with the quin-2 fluorescence result (2.032 s\(^{-1}\)), this peptide does not seem to affect the N-lobe calcium dissociation rate.

3.4.8 Tyrosine fluorescence of RC3 (13-52) peptide with CaM

Figure 3.17: Fluorescence due to calcium release from CaM in the presence of RC3 (13-52) peptide measured using intrinsic tyrosine fluorescence
Discussion of RC3 (13-52) peptide interaction with CaM

In an interesting result, tyrosine fluorescence of the RC3 (13-52) peptide indicates that this peptide accelerated calcium dissociation from the C-terminal lobe and hence one of the rates of $10.66 \text{ s}^{-1}$ observed with quin-2 fluorescence cannot simply be assigned to C-terminal lobe. The result with this peptide seems anomalous in trying to determine its effect on calcium dissociation from CaM. As this thesis was being written, experiments conducted by others in our lab with this peptide suggested that the peptide was contaminated. The purified peptide showed different results. Hence, it appears that one of the two rates obtained using quin-2 fluorescence was an artifact.

Table 3.3: Summary of kinetics of calcium dissociation from CaM in the presence of neurogranin peptides

<table>
<thead>
<tr>
<th>Peptide/Protein</th>
<th>Rate (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaM only</td>
<td>12.93</td>
</tr>
<tr>
<td>CaM with RC3(26-49) peptide</td>
<td>2.032</td>
</tr>
<tr>
<td>CaM with RC3(13-49) peptide</td>
<td>447.25</td>
</tr>
<tr>
<td>CaM with RC3(13-52) peptide</td>
<td>138.30</td>
</tr>
<tr>
<td></td>
<td>10.66</td>
</tr>
</tbody>
</table>

Experimental measurement: Quin-2 fluorescence

<table>
<thead>
<tr>
<th>Peptide/Protein</th>
<th>Rate (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CaM only</td>
<td>11.67</td>
</tr>
<tr>
<td>CaM with RC3(26-49) peptide</td>
<td>2.83</td>
</tr>
<tr>
<td>CaM with RC3(13-49) peptide</td>
<td>285.79</td>
</tr>
<tr>
<td>CaM with RC3(13-52) peptide</td>
<td>179.05</td>
</tr>
</tbody>
</table>

Experimental measurement: Tyrosine fluorescence
3.4.9 Summary and discussion

The results show that the IQ domain region of neurogranin alone is not sufficient to mimic the entire protein. Additional residues at the C-terminal and N-terminal end of the IQ domain significantly impact the behavior of the peptides. Another result from the series of experiments is that an aspartate at the serine 36 position does not mimic phosphorylation of the residue. These are important results to bear in mind while using neurogranin peptides to study the behavior of this protein.

Another result with biological implications is the behavior of different SNIQ domain proteins in regulation of CaM. From the peptide studies, it is clear that the different regions of PEP-19 and neurogranin are functionally similar in terms of their ability to accelerate or decelerate calcium off-rates. However, PEP-19 in addition to accelerating calcium off-rates also accelerates calcium on-rates (X. Wang et al. 2010) whereas neurogranin accelerates only calcium off-rates (Gaertner, Putkey, and Waxham 2004). In the brain, PEP-19 is primarily expressed in the cerebellar purkinje cells whereas neurogranin is expressed in the CA1 and CA3 regions of the hippocampus and regions of the frontal cortex. Both these proteins regulate CaM’s ability to transduce calcium signals by acting as a calcium buffer. Experiments in the hippocampal regions of rat brain suggest that regulation of postsynaptic calcium through modulation of extrusion and/or buffering regulates expression of long-term potentiation in the CA2 and potentially other brain regions(Simons et al. 2009). Since the cerebellum and hippocampus have different calcium dynamics, it is likely that neurogranin and PEP-19 evolved to specifically handle calcium signaling in that region of the brain.
CHAPTER 4

GENERAL DISCUSSION
4.1 Overview

In the final section of my thesis, I will describe the role of neurogranin as a regulator of the calcium transducing protein, CaM based on my quantitative study of the binding affinity of neurogranin peptides with CaM and the effects of these peptides on the kinetics of calcium binding to CaM. This quantitative study has helped delineate the role of neurogranin on the time-dependent distribution of CaM and calcium bound CaM following calcium influx into neurons and the equilibrium concentrations of these species.

The emerging story on neurogranin is that like other members of the small neuronal IQ domain protein (SNIQ) family, this protein serves to alter the kinetics of calcium binding to CaM which in turn fine tunes calcium signaling following calcium influx into the neuron. As is evident from the data reported in literature, the kinetics of calcium influx and signaling following this event are in the order of a few milliseconds and involve a few micromolar of calcium influx ((Spruston et al. 1995; Sabatini, Oertner, and Svoboda 2002; Maravall et al. 2000)). In order to transduce a calcium signal of such short duration and strength, the spatial and temporal distribution of CaM is critical.

I used peptides of neurogranin that represent different regions of the neurogranin protein in my experiments. In the first section, I will address the issue of the peptide that best mimics the protein by comparing the thermodynamic binding affinity results from the peptides and the protein. I will next address the effect that each of the neurogranin peptides have on calcium binding to CaM by calculating the free energy of calcium binding to CaM in the presence of each of the peptides and calculating the dissociation constant from the free energy value. Finally, a computational model of CaM interaction with neurogranin and
calcium was built using MATLAB. Using the binding affinity information obtained from calorimetry experiments and the calcium binding kinetics data obtained from the fluorimetry data, I have constrained the parameters of the model. In the last section of discussion, I address the effect of neurogranin on the concentration of calcium-saturated CaM which is the CaM species that activates downstream targets.

4.2 Characterization of domains of neurogranin required for its function

To characterize the functional domains of neurogranin, peptides encompassing different regions were synthesized. In selecting regions of neurogranin critical for its function, the IQ domain region is the focus due to sequence homology between the IQ regions of neurogranin (RC3), GAP 43 and PEP 19 (see figure below). I used three different peptides each of which contained the IQ region of the protein along with residues at either the N-terminal or the C-terminal which have been suggested to play an important functional role.

The three peptides are: RC3 (26-49) which is the shortest peptide containing the IQ region and a few residues N-terminal to it, RC3 (13-49) which encompasses the IQ region and acidic residues N-terminal to it which are believed to play a critical role in functional interaction with CaM and RC3 (13-52) the longest peptide synthesized. I also purified the neurogranin (RC3) protein and used it in my experiments.
To determine the peptide that best mimics the protein, I determined binding affinities using isothermal titration calorimetry. The experiment was performed as described in the materials and methods section. The results indicate that the RC3 (13-49) peptide interacted with apo-CaM with an affinity of 0.066 μM and bound to calcium-CaM with an affinity of 1.25 μM. Thus, functionally the RC3 (13-49) peptide mimics the protein itself which bound to apo-CaM with an affinity of 0.071 μM and to calcium-CaM with an affinity of 1.39 μM. These results align with the properties of PEP-19 peptides where the peptide containing the IQ domain and an acidic domain N-terminal to it functionally mimics the full protein (Putkey et al. 2008).
To mimic PKC phosphorylation, we used two peptides with modifications to the serine residue in the IQ domain of RC3. The RC3 S36D peptide has a serine residue that is replaced by aspartate that imparts a negative charge at that position similar to a phosphorylated residue. The RC3 S36P residue has a phosphorylated serine at the 36th position instead of serine.

The ITC results indicate that while the affinity of RC3 S36D peptide for apo-CaM is 2.29 μM (number of trials=2) and affinity towards calcium-CaM is 5.27 μM (number of trials=2), the affinity of RC3 S36P peptide for CaM is below detection.
Table 4.2: Binding affinities for peptides with phospho modifications at serine residue

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Interaction with apo-calmodulin</th>
<th>Interaction with calcium-calmodulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3 S36D</td>
<td>N- 1.09</td>
<td>N- 0.95</td>
</tr>
<tr>
<td></td>
<td>$K_d$- 2.29 uM</td>
<td>$K_d$- 5.27 uM</td>
</tr>
<tr>
<td>RC3 S36P</td>
<td>Below detection</td>
<td>Below detection</td>
</tr>
</tbody>
</table>

The ITC results with RC3 S36P and RC3 S36D indicate that the RC3 S36D peptide does not mimic phosphorylation of serine at the 36th position. In literature, the RC3 S36D peptide has been used as a phospho-mimetic (Gerendasy et al 1994). This is an important conclusion from our work on the characterization of domains of neurogranin required for its function.

4.3 Free energy of calcium binding to CaM in the presence of neurogranin peptides and protein

Free energy of calcium binding to CaM in the presence of the various neurogranin peptides can be calculated using a scheme similar to the one used by Storm and colleagues for calculation of calcium binding to CaM in the presence of troponin I (TnI) (Keller et al., 1982).
Figure 4.2: Scheme for calculation of free energies of calcium and neurogranin peptide binding to CaM

Applying the principle of conservation of free energy to the scheme above,

\[ 4\Delta G^\circ(C) = \Delta G^\circ(R/C_4) - \Delta G^\circ(R) + 4 \Delta G^\circ(C) \]

Free energies can be calculated using the formula

\[ \Delta G^\circ = RT\ln(K_d) \]

\( \Delta G^\circ(R/C_4) \) is the free energy of neurogranin binding to calcium/CaM, \( \Delta G^\circ(R) \) is the free energy of neurogranin binding to apo-CaM, \( \Delta G^\circ(C) \) is the free energy of calcium binding to CaM. \( \Delta G^\circ(C) \) is calculated from the dissociation constant values reported in literature. \( \Delta G^\circ(R/C_4) \) and \( \Delta G^\circ(R) \) values are calculated from the dissociation constants measured for each of these peptides from ITC measurements.

The calculated values are reported in the table below and the last column represents the dissociation constant for calcium binding to CaM in the presence of each of these.
peptides/protein. From the table below, the conclusion is that when RC3 (26-49) peptide is present, the calcium affinity of CaM is twice as high when compared to the calcium affinity of CaM in the presence of neurogranin protein or any of the peptides that contain the acidic domain in addition to the IQ domain of neurogranin. Furthermore, the full protein and the RC3 (13-49) peptide are identical in their ability to alter calcium affinity toward CaM based on the dissociation constant values. The RC3 (13-52) peptide bound to CaM also shows a very similar dissociation constant to both the full protein and RC3 (13-49) peptide indicating that the acidic residues N-terminal to the IQ domain along with the IQ domain are important for the function of the protein.

Table 4.3

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>$\Delta G^\circ (R/C_4)$ Kcal/mol</th>
<th>$\Delta G^\circ (R)$ Kcal/mol</th>
<th>$\Delta G^\circ (C)$ Kcal/mol</th>
<th>$\Delta G^\circ (C/R)$ Kcal/mol</th>
<th>$K_d$ (C/R) $\mu$M</th>
</tr>
</thead>
<tbody>
<tr>
<td>RC3(26-49) bound to CaM</td>
<td>-0.08</td>
<td>-0.27</td>
<td>-1.02</td>
<td>-0.97</td>
<td>0.19</td>
</tr>
<tr>
<td>RC3(13-49) bound to CaM</td>
<td>0.13</td>
<td>-1.60</td>
<td>-1.02</td>
<td>-0.59</td>
<td>0.37</td>
</tr>
<tr>
<td>RC3(13-52) bound to CaM</td>
<td>0.50</td>
<td>-1.08</td>
<td>-1.02</td>
<td>-0.625</td>
<td>0.35</td>
</tr>
<tr>
<td>RC3 protein bound to CaM</td>
<td>0.19</td>
<td>-1.56</td>
<td>-1.02</td>
<td>-0.58</td>
<td>0.37</td>
</tr>
</tbody>
</table>
4.4 Simulations of the effects of neurogranin on calcium saturation of CaM and putative mechanism of neurogranin interaction with CaM

To study the effect of neurogranin on the concentrations of different species of CaM (apo-CaM, partially saturated CaM, saturated CaM), I ran simulations in MATLAB. In the first simulation I wanted to study the effect of neurogranin on calcium binding to CaM. One of the salient features of calcium binding to CaM is that upon calcium influx, the N-lobe of CaM initially saturates with calcium following which calcium redistributes to the C-lobe of CaM. This occurs within the first few milliseconds and the effect is best studied using simulations. I simulated calcium binding to CaM in the absence of neurogranin by running the simulation with a CaM concentration of 21 µM, a sub-saturating calcium concentration of 56 µM and followed the concentrations of N-lobe saturated CaM, C-lobe saturated CaM and fully saturated CaM. The figure below is the simulation result.

What I saw was that the N-lobe of CaM (shown in green) was quickly saturated with calcium and showed an increase in concentration. This was followed by a redistribution of calcium to the C-lobe of CaM which resulted in a slower rise in the concentration of C-lobe saturated CaM and fully saturated CaM.
I then added 21 µM neurogranin to the simulation and noticed that calcium binding to CaM followed the same pattern indicating that neurogranin does not change the mode of calcium binding to CaM. However, neurogranin reduces the equilibrium concentration of calcium saturated CaM as well as the concentration of the other species of CaM.
After establishing that neurogranin does not change the mode of calcium binding but reduces the equilibrium concentration of CaM species, I chose to look at four different species namely, CaM, neurogranin-bound CaM, fully calcium saturated CaM, fully saturated neurogranin bound CaM.

The simulation results show that neurogranin quickly complexes with CaM and upon calcium influx the concentration of CaM in complex with neurogranin binds to a larger proportion of calcium than un-complexed CaM. This is an interesting and unexpected result because neurogranin accelerates calcium release from CaM. Hence, the prediction was that...
there would be a transient increase in saturation of neurogranin complexed with CaM followed by a drop in the concentration. At first pass, this result suggests that the concentration of neurogranin-bound CaM is extremely high and serves to drive the reaction in a direction that is kinetically unfavorable. In an attempt to better understand this result as well as dissect the mechanism by which the calcium saturated states of CaM and neurogranin bound CaM are populated, I looked at the concentrations of the partially saturated CaM and neurogranin-bound CaM species.

**Figure 4.5: Concentrations of CaM and Neurogranin bound CaM species**

A look at the partially saturated species of CaM and neurogranin CaM showed that neurogranin bound CaM species was short-lived. In combination with the earlier result, this suggests that neurogranin bound CaM is quickly saturated with calcium which is extremely
unfavorable kinetically. When considering that the rate of decay of the partially saturated species to its apo-form is 30 fold higher than the rate of generation of calcium–saturated CaM species, the conclusion is that for such a kinetically unfavorable reaction to proceed, the driving force must be exceptionally high. The driving force is the concentration of neurogranin.

Figure 4.6: Concentrations of partially saturated CaM and Neurogranin bound CaM species
Finally, I studied the effect of neurogranin on the equilibrium concentrations of CaM and neurogranin-bound CaM. I repeated this simulation with two different concentrations of calcium influx.
Figure 4.8: Equilibrium concentrations of CaM and Neurogranin/CaM species with 56 µM calcium influx
The results show that above a certain concentration of neurogranin (a sub-equimolar concentration), neurogranin bound CaM is primarily calcium saturated. Below that concentration, the concentration of saturated CaM dominates. This result has implications for the biological role of neurogranin as post-translational modifications like phosphorylation reduce the concentration of neurogranin available to bind to CaM which could influence the population of the calcium saturated state of CaM.
4.5 Conclusions and Future Experiments

In this thesis, I studied the effect of neurogranin on calmodulin’s ability to interact with calcium. I used biophysical techniques like isothermal titration calorimetry to measure the binding affinity between peptides representing different domains of neurogranin and calmodulin and stopped-flow fluorimetry to measure calcium dissociation from calmodulin in the presence of neurogranin peptides. I also performed simulations in MATLAB to predict the effect of the peptides on calcium saturation of CaM.

From my results, the overall conclusions are

• The minimal IQ domain of neurogranin (RC3(26-49)) does not mimic the full protein

• The IQ domain along with acidic residues N-terminal to it (RC3(13-49)) mimics the protein best

• Replacement of serine residue within the IQ domain of neurogranin with aspartate does not mimic phosphorylation of this residue

• Neurogranin exerts its effect on calcium saturation of CaM through a mechanism that is concentration dependent such that it drives it in a kinetically unfavorable direction. This result is relevant when you consider that phosphorylation of neurogranin by PKC prevents its interaction with CaM and hence reduces the functionally relevant concentration of neurogranin.
Future experiments would involve studying the effect of neurogranin on calcium interaction with calmodulin following low-frequency stimulations that result in long-term depression and high-frequency stimulations that result in long-term potentiation. This would require simulations in MATLAB that measure concentrations of calcium saturated CaM following calcium influx after a high-frequency stimulation or a low-frequency stimulation. Another issue to study would be the effect of phosphorylation of neurogranin on calcium interaction with CaM in a systematic way. To do this, currently known experimental values would be used to obtain optimal parameters. Using the optimal parameters, a model for phosphorylated neurogranin interaction with CaM would be built and the effect of high-frequency and low-frequency stimulations would be studied.
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