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**Targeting of Calcium/Calmodulin-Dependent
Protein Kinase II to Membranes**

by

Shahriar Saeedi, B.Sc. (Hons.)

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of
Masters of Science

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submitted by

Shahriar Saeedi, B.Sc. (Hons.)

In partial fulfillment of the requirements for the degree of Masters of Science

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ABSTRACT

Communication between neurons is mediated by the controlled release of neurotransmitters from small synaptic vesicles. Several proteins are involved in the regulation of neurotransmitter release. One such family of proteins is the calcium/calmodulin-dependent protein kinases. Calcium/calmodulin-dependent protein kinase II (CaMKII) is one of the most abundant members of this protein family, and is found in many tissues with specific isoforms usually predominating in certain tissues. CaMKII is found co-purified with synaptic vesicles. The question of how CaMKII is attached to the synaptic vesicle is addressed in this thesis. It was hypothesized that CaMKII may interact with the phospholipid component of the vesicle membrane, which could mediate binding.

C-terminal deletion mutants of murine α -CaMKII were produced by PCR directional cloning and liposome-binding assays were performed on the purified proteins. Binding of murine α -CaMKII to several types of liposomes was not observed. Recent studies into targeting of this enzyme suggest a role for anchoring proteins. Murine α -CaMKII like the β isoform from rat muscle may interact with membranes through a putative non-kinase translation product (a putative anchoring protein), which encompasses amino acids 230-270 at N-terminal of the regulatory domain rather than through direct binding with phospholipids.

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
AKAP	A kinase anchoring protein
AOX	Alcohol oxidase
α KAP	α Kinase anchoring protein
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazole-propionate
BSA	Bovine Serum Albumin
BB	Breaking buffer
BCA	Bradford-Coomassie assay
Δ C	C-terminal deletion
CaM	Calmodulin
CaMK	Ca ²⁺ /Calmodulin Kinase
CPG	Controlled pore glass
CRE (B)	cAMP Response element (binding protein)
DOPC (S)	Dioleoyl phosphatidyl choline (serine)
DTT	1,4-Dithiothreitol
ECL	Enhanced chemiluminescent
EDTA	Ethylenediamine tetraacetic acid
GST	Glutathione S-transferase
HEPES	(N- [2-Hydroxymethyl] piperazine-N'-[2-ethanesulfonic acid])
HRP	Horseradish peroxidase
IPTG	Isopropyl α -D-thiogalacto-pyranoside

LB	Luria broth
LTD	Long-term depression
LTP	Long-term potentiation
MLV	Multilamellar vesicle
NMDA	N-methyl-D-aspartate
PCI	Phenol chloroform isoamyl alcohol
PCR	Polymerase Chain Reaction
PKA	Protein kinase A
PKC	Protein kinase C
PMSF	Phenyl-methyl-sulfonyl fluoride
PSD	Post synaptic density
RACK	Regulatory domain associated of C kinase
RPM	Round per minute
SCH	Southern Colony Hybridization
SDS	Sodium Dodecyl Sulfate
SSPE	Sodium chloride sodium phosphate-EDTA
SV	Synaptic vesicle
SYN 1	Synapsin I
TE	Tris-EDTA
TEMED	N, N, N', N'-tetramethyl-ethylenediamine
YPD	Yeast peptone dextrose

Chapter 1.

Introduction

1. INTRODUCTION

Signaling and intracellular information processing in the brain is highly complex. To accomplish these tasks, neurons possess a vast array of signaling mechanisms such as protein phosphorylation/dephosphorylation and second messenger systems, such as those involving calcium (Ca^{2+}). Studies initiated in the late 1980s established that the serine/threonine protein kinase, calcium/calmodulin-dependent protein kinase II (CaMKII), is one of the general protein kinases of the Ca^{2+} signaling systems (Figure 1.1). Multifunctional CaMKII is highly concentrated in brain (particularly in cortical and hippocampal structures) and is localized on both sides of synapses. CaMKII is a necessary component in the induction of long-term potentiation (LTP), a mechanism for learning and memory, and maintains its activity by autophosphorylation (of neighboring CaMKII and/or itself) after activation by a brief Ca^{2+} signal (Hanson and Schulman, 1992; Pettit *et al.*, 1994; Fukunaga *et al.*, 1995). Autophosphorylation at threonine²⁸⁶, in the presence of Ca^{2+} /calmodulin, generates a Ca^{2+} -independent form of the protein which prolongs the effects of transient Ca^{2+} elevation *in vivo* (Lisman, 1994).

CaMKII has substrates such as the synaptic vesicle-associated protein, synapsin I, in the synaptic nerve terminal, and AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole-propionate) channels in the post-synaptic membrane. CaMKII is also involved in Ca^{2+} -stimulated transcription of CRE-containing (cAMP Response-Element) genes through phosphorylation of the CREB (cAMP Response-Element Binding protein) transcriptional activator via phosphorylation of CaMKIV, a nuclear homologue of the protein (Heist and Schulman, 1998).

1.1 Biochemical and Structural Characteristics of CaMKII

The rat brain CaMKII is an enzyme comprised of 10 to 12 subunits with three isoforms in a ratio of 3-4:1: α (54 kDa), β and/or β' (55 and 50 kDa respectively). The α and β isoforms are encoded by separate genes, whereas the β' isoform is a splice variant of the β isoform (Schulman, 1993). Additional isoforms such as the γ and δ , which are expressed in brain and other tissues, show approximately 85% amino acid and 75% nucleotide identity to the α isoform (Hanson and Schulman, 1992, Brocke *et al.*, 1999). Proteins in this family differ in variable regions at the C-terminal end of the calmodulin-binding domain (Bulliet *et al.*, 1988).

α/β CaMKII can be co-purified with synaptic vesicles (SV) through controlled-pore glass (CPG) chromatography and represents 2% of the total vesicle-associated proteins (Benfenati *et al.*, 1992; Greengard *et al.*, 1993). CaMKII associates with SV membranes even at ionic strengths that produce quantitative dissociation of synapsin I. Biochemical and functional characterization of SV-associated CaMKII showed that it has similar properties to its soluble counterpart (Benfenati *et al.*, 1996). In addition, Bayer *et al.* (1998) indicated that CaMKII isoforms may be anchored to the membrane via their N-terminal hydrophobic domain and co-assemble with an anchoring protein named α KAP (alpha kinase anchoring protein) *in vivo* (Bayer *et al.*, 1998).

The α -CaMKII gene encodes 478 amino acids in a 5.0 kilobase message. α -CaMKII is comprised of an N-terminal catalytic domain where phosphorylation sites are located (residues 1 to 263-274), a C-terminal oligomerization domain (i.e. association domain, residues 311-478), and a regulatory domain (residues 275-310) in between the above mentioned domains (Figure 1.2). The regulatory domain of α -CaMKII consists of

two motifs which overlap, a calmodulin (CaM) binding domain (residues 296-309) and an autoinhibitory domain (residues 281-302) that interacts with the catalytic domain and inactivates the kinase (Cruzalegui *et al.*, 1993). Electron microscopy of rat forebrain CaMKII suggests a flower-with-petals structure with the association domains forming a globule in the center and the catalytic domains radiating from it (Kanseki *et al.*, 1991; Schulman, 1993) (Figure 1.3).

CaMKII is inactive in the absence of Ca^{2+} /calmodulin (Dupont and Goldbeter, 1998). Attachment of four calcium ions to calmodulin induces a conformational change that increases the affinity of calmodulin for CaMKII (Schulman, 1993). CaMKII is activated at a Ca^{2+} /calmodulin concentration between 25 and 100 nM and its affinity is increased at higher ATP concentrations (De Koninck and Schulman, 1998). Calmodulin wraps around the autoinhibitory sequence of CaMKII, which in turn locks itself in an activated state through autophosphorylation of threonine²⁸⁶ (in α -CaMKII) to produce a partially active kinase (Ikura *et al.*, 1992; Meador *et al.*, 1992).

The regulatory domain of CaMKII is bifunctional, in the sense that it is needed for both calmodulin binding and autoinhibition of the kinase (Cruzalegui *et al.*, 1993). The use of CaMKII inhibitors (e.g. KN62/93) has shown that inhibition of the enzyme is competitive with ATP and non-competitive with peptide substrates (e.g. Synapsin I). Once Ca^{2+} /calmodulin is bound, autophosphorylation of threonine²⁸⁶, allows the kinase to continue phosphorylation in a Ca^{2+} -independent fashion (Figure 1.3). Furthermore, replacement of threonine³⁰⁵ and threonine³⁰⁶ inhibitory sites with alanine or leucine showed that the autophosphorylation of either of these two sites is sufficient for inhibition of calmodulin-stimulated activity (Schulman, 1993) (Figure 1.3). A model of

CaMKII regulation in Figure 1.3 shows how by autophosphorylation and Ca^{2+} /calmodulin-trapping allows the α -CaMKII holoenzyme to recruit more calmodulin during successive Ca^{2+} spikes. This may imply that CaMKII is involved in some form of molecular memory storage process and may work as a molecular switch (Lisman *et al.*, 1997; De Koninck and Schulman, 1998).

1.2 Major Functions of CaMKII in Neurons

1.2.1 Role of CaMKII in neurotransmitter release

The first step in the Ca^{2+} -dependent process of neurotransmitter release is to free synaptic vesicles from actin and other cytoskeletal elements (Bajjalieh and Scheller, 1995). Synapsin I is phosphorylated by α -CaMKII and consequently loses its affinity for synaptic vesicles due to conformational changes that causes the release of actin filaments (Benfenati *et al.*, 1996). Vesicles released by phosphorylated synapsin I then move to the active zone where they are ready for exocytosis. A model of the interaction of synapsins and their phosphorylation by CaMKII is presented in Figure 1.4. Recent results suggest that dynamic association of synapsins with synaptic vesicles correlates with their role in activity-dependent plasticity through a novel mechanism by which proteins reversibly bind to membranes using a phosphorylation-dependent phospholipid-binding domain (Hosaka *et al.*, 1999).

1.2.2 Role of CaMKII in synaptic plasticity, learning and memory

Long lasting increases and decreases in synaptic strength and response to different patterns of synaptic activation are thought to form the basis for the neuronal changes that contribute to learning and memory (Silva *et al.*, 1992). LTP (long-term

potentiation) and LTD (long-term depression) are the best-studied examples of activity dependent modifications that may underlie learning and memory in the CA1 region of the hippocampus, important for the storage of explicit forms of memory (Glazewski *et al.*, 1996).

Both LTP and LTD can be induced at the same synapses by different frequencies of stimulation; low frequencies induce LTD, whereas high frequencies induce LTP (Mayford *et al.*, 1995; Mayford *et al.*, 1996). Although the molecular basis of these two forms of plasticity is unclear, both require influx of Ca^{2+} into the postsynaptic cell through N-methyl D-Aspartate (NMDA) receptors (Lisman *et al.*, 1997).

The ability of CaMKII to convert itself into a Ca^{2+} -independent state following repetitive exposures to Ca^{2+} has led to the suggestion that the protein functions as a memory molecule in LTP. Moreover, mice lacking the α isoform of α -CaMKII also lack LTP and have a deficit in spatial learning (Silva *et al.*, 1992; Giese *et al.*, 1995). There is now considerable evidence that the enhanced efficacy of synapses after LTP is due, in part, to postsynaptic changes (Rotenberg *et al.*, 1996). In glutamergic excitatory synapses, which are the most extensively studied of synapses, induction of LTP enhances the postsynaptic response to glutamate. Activation of α -CaMKII in PSD (Post-Synaptic Density) by Ca^{2+} influx through NMDA glutamate receptor/ion channels activates the PSD-CaMKII which, in turn, phosphorylates AMPA channels enhancing their subsequent responsiveness to released glutamate (Lisman, 1994).

1.2.3 Localization of CaMKII

Neuronal signaling requires that synaptic proteins such as CaMKII be appropriately localized within the cell. CaMKII is 20-50-fold more concentrated in the

brain particularly in the hippocampus, amygdala, cortex, striatum, and lateral septum than in non-neuronal tissues (Wu *et al.*, 1996; Wu *et al.*, 1999). The subcellular levels of CaM kinase in neurons vary for different parts of the cell. Different isoforms of CaM kinase are found as soluble forms in the cytosol or in particulate fractions, including PSD. α -CaMKII is particularly concentrated in the PSD (total concentration of 20-40% of all PSD proteins) where it is associated with NMDA channels (Leonard *et al.*, 1999; Shen and Meyer, 1999).

Numerous questions remain about CaMKII action and function in brain. One of these is: how is α -CaMKII bound to the synaptic vesicles? The hypothesis tested in the present study is that the variable region (a linker region which differs in various CaMKII isoforms and is located between regulatory and association domains, see Figure 1.2) of the protein has a prominent role in binding the enzyme to synaptic vesicle membranes.

To test this hypothesis, a series of C-terminal deletion mutants (encompassing the variable region) of murine α -CaMKII were produced using directional cloning. The cloned cDNAs were then used to express the recombinant proteins in both eukaryotic (*Pichia pastoris*) and prokaryotic (*Escherichia coli*) expression systems followed by protein purification using appropriate affinity chromatography methods (GST-fusion or 6 $\times$ his-tagged). The purified proteins were then used in binding assays to test their affinities for lipid membranes.

Figure 1.1 Cross talk between CaMKIIs in Ca²⁺ signaling systems.

Most eukaryotic cells possess protein kinases that are stimulated by a complex of calcium and the calcium-binding protein calmodulin. These kinases are referred to as calcium/calmodulin-dependent protein kinases (CaM kinases). Many CaM kinases such as CaMKII function in the cytoplasm. When calcium enters the cell, it binds with high affinity to calmodulin. The calcium/calmodulin complex then binds to a CaM kinase and activates it. CaMKII phosphorylates several substrates, including itself. In addition, CaM-dependent kinases also interact with other protein kinases such as PKA and/or PKC (not included in diagram).

Figure 1.2 The structure of multifunctional CaMKII

A. The ribbon diagram of the enzyme complexed, with Ca^{2+} /calmodulin was obtained by superimposing other kinases with known structures (e.g. 1ATP.pdb for PKA) and incorporating mutagenesis data from Cruzalegui *et al.*, (1993). The C-terminal domain and ATP binding region are located close to the active site in the middle of structure. CaMKII is present in neurons as an oligomer (10 to 12 subunits). **B.** Domains of α -CaMKII. CaMKII is composed of three domains, an N-terminal catalytic domain, a central regulatory domain, and a C-terminal association domain. The N-terminal domain mediates phosphorylation of substrates. The regulatory domain binds calcium/calmodulin and possesses an auto-inhibitory site. The association domain mediates interaction between CaMKII monomers, and interactions with other proteins (Soderling, 1993).

B

Figure 1.3 Role of CaMKII in the synaptic vesicle cycle.

When an action potential reaches the nerve terminal, voltage-gated calcium channels open allowing a rapid influx of calcium ions which activate several processes. One such process is the fusion of synaptic vesicles with the plasma membrane, which releases neurotransmitter. Calcium also binds to calmodulin, which in turn activates several protein kinases such as CaM kinase II. CaM kinase II phosphorylates a variety of substrates including synapsin I (which is the best substrate for CaM kinase II so far). The phosphorylation of synapsin I by CaM kinase II occurs rapidly and to high stoichiometry during physiological activity of neurons. Phosphorylation by CaM kinase II causes a conformational change in the synapsin I molecule, and is associated with major changes in its biological properties. Upon phosphorylation of synapsin I, vesicles can be released from actin filaments and move from a non-releasable pool of vesicles into a releasable pool (Bennett and Scheller, 1994; Südhof, 1995).

Figure 1.4 Model for synaptic regulation by CaMKII

A. The catalytic subunit is represented as a large sphere, and the association domain as a small sphere. The regulatory domain is shown as a rod connecting the other two domains. When calcium bind to calmodulin, the complex can then bind to CaMKII leading to removal of the auto-inhibitory domain from the active site of the enzyme, and subsequent enzyme activation. The active enzyme can then phosphorylate substrates, including itself. **B.** When the enzyme is activated by calcium/calmodulin, the CaMKII monomers can phosphorylate their neighboring monomer in the CaMKII oligomer. This autophosphorylation leads to continued activation of the enzyme, even in the absence of calcium.

Chapter 2.

Methods & Materials

2. METHODS AND MATERIALS

2.1 Cloning and expression of murine α -CaMKII using bacterial GST fusion vectors GEX2T/4T1.

2.1.1 Plasmid and Bacterial Strains

GST fusion plasmids pGEX2T and pGEX4T1 were used in the following experiments. These plasmids offer features such as a *tac* promoter for chemically inducible, high level expression, an internal *lacI*^q gene for use in any *E. coli* host, very mild elution conditions for release of fusion proteins from the affinity matrix, and Xa or thrombin proteolytic cleavage sites for removal of the fusion partner.

E. coli DH5 α (F⁺lendA1 hsdR17 (r_k⁻m_k⁺) supE44 thi-1 recA1 gyrA (Nal^r) rel A-1 Δ (lacIZYA-argF) U169 deoR (ϕ 80 dlac Δ (lacZ) M15) and *E. coli* JM103 (F⁺tra D36 lacIq Δ (lacZ) M15 proA+B+/end A1 supE sbcBC thi-1 rpsl (str^r) Δ (lac-pro)(p1) (r_k⁺m_k⁺ r_{pl}⁺m_{pl}⁻), were used as host strains for electroporation. *E. coli* BL21 (DE3) (F⁻ ompT [lon] hsdSB (r_B⁻m_B⁻) was used as expression host. Media used in course of experiments: 20g/L LB(Nutrient broth 1 or 2 OXOID Inc.)-agar media or 20g/l LB + 15 g/l agar-agar (BDH Inc.) containing 100 μ g/ml of ampicillin.

2.1.2 Cloning

Complementary DNA coding for murine α -CaMKII was subjected to PCR using primers harboring *Bam*H1 and *Eco*R1 restriction sites (1960-TAATGAATTCTTTGCGACTTTGTGTCTT, 2043-TGTCGATTCGGGTCG CACATCTTCGTGTA, 2126-TTCCGAATTCACAAGTTTTCAAATAGAAT, 2200-AGGCGAATTCGGCTGACTCGTCACCCATCA as forward primers and 925-

CGCGTGGATCCCCCATGGCTACCATCACCT as the sole reverse primer) PCR amplification of genes was performed according to the following protocol. Ten μ l of 10X PCR buffer without Mg^{2+} [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 2 μ l of dNTPs 10 mM (total of 0.02 μ moles, final concentration of 0.2 mM), 10 μ l of each reverse and forward primers (10 mM), 2 μ l of 0.012 μ g/ μ l pGEX2T/4T-1 stock in TE [1 mM Trizma base, 1mM EDTA sodium salt (pH 8.0) (total of 20ng), 3 μ l of 50 mM $MgCl_2$ and 0.5 μ l of Taq DNA polymerase (5 units/ μ l) were added to 0.2 ml eppendorf tube, containing 62.5 μ l of ddH₂O. After pre-dwelling for 1 minute at 94°C, 25-30 PCR cycles were run according to the regime in an AMPLITRON II: denaturing at 94°C for 1 minute, annealing at 60°C for 30 sec and extension at 72°C for 1 minute per cycle.

PCR products were purified using the Wizard PCR purification system (Promega) and further purified using PCI extraction before digestion. Fifty μ l (0.5 μ g/ μ l) of PCR products, 8 μ l of react buffer 3 (Gibco-BRL), 10 units of each *Bam*HI and *Eco*RI (GIBCO-BRL) were added to 0.5 ml tubes containing 12 μ l of ddH₂O bringing the volume to 90 μ l. Tubes were incubated at 37°C for 3-4 hrs and PCI extracted the day after. The concentration of DNA was determined at an optical density of 260 nm (OD_{260}). Vectors pGEX2T/4t-1 were also digested using the same method.

One and half pmoles of digested PCR products and 0.5 pmole of pGEX vectors were added to 0.5 ml Eppendorf tubes and the volumes were brought up to 9 μ l with 1 μ l of 1X DNA ligase buffer [50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 μ g/ μ l BSA], and the appropriate amount of ddH₂O. Ligation mixtures were spun for 20 sec and were incubated in a 60°C water bath for 1 minute, then cooled down

on ice and 1 µl of DNA T4 ligase (New England Biolabs) was added and incubated at 4°C overnight. One µl of each sample was used to transform host *E. coli* strains.

2.1.3 Diagnostic analysis

Bacterial transformants were screened using Southern colony hybridization and/or diagnostic restriction analysis followed by automated DNA sequencing (Canadian Molecular Research Services Inc.) For Southern colony hybridization, a hybridization solution was made as follows. Twenty five µl of [γ 32P]- labeled primers were added to a mixture of 150 ml ddH₂O, 625 µl of 20X SSPE, 25 µl of 100X Denhardt's reagent (2% Bovine Serum Albumin [BSA], 2% Ficoll and 2% polyvinylpyrrolidone [PVP] -360), 12.5 ml of SDS 10% and 500 µl of herring sperm DNA. After colonies were transferred to nitrocellulose membranes, cells were lysed on a piece of filter paper with lysis solution (1.5 M NaCl and 0.5 M NaOH), neutralized with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl pH 7.2), fixed under UV light for 15 min and incubated in hybridization solution at T_m (dependent on the primers, around 50°C) in revolving incubators for 12-18 hrs. Tubes were subjected to successive stronger stringency with 2×, 1× and 0.1× SSPE/SDS washing solutions (1-2°C>T_m). Positive clones were selected according to radiograph and checked for the expected plasmid sizes by 0.6% agarose gel profiling.

2.1.4 Batch and column expression/purification of GST fusion proteins

The following protocol was employed in production of GST fusion α -CaMKII and its C-terminal mutants from 100 ml cultures of *E. coli* BL21 containing pGEX vectors carrying appropriate cDNAs. Cultures were allowed to grow at 37°C to OD₆₀₀ $\approx$ 0.5, induced with a final concentration of 0.1-0.2 mM IPTG (Isopropyl β -D-Thiogalacto-

pyranoside), and then incubated for an additional 1-4 hrs. Cells were pelleted at $5000 \times g$ at 4°C and were resuspended in 10 ml of ice-cold resuspension solution ($1 \times \text{HBS} / \text{PBS}$, pH 7.4). The solutions were then either sonicated or lysed with B-PER bacterial lysis solution [a mild nonionic detergent (proprietary Pierce Inc.) in 20 mM Tris-HCl pH 7.5] and centrifuged at $7000 \times g$ at 4°C for 10 min. One ml of 50% slurry of glutathione Sepharose-4B beads (Pharmacia) was added to the supernatant and gently mixed at 4°C in a cold room for 30-60 min. At this step slurry mixtures were either transferred to a chromatography column (BIO-RAD 0.8×4 cm) or washed with $1 \times \text{HBS/PBS}$ in an Eppendorf tube for batch purification. For column purification, the beads were washed with 5-10 $\times$ bed volumes of $1 \times \text{HBS/PBS}$, and fusion proteins were eluted with 0.5-1 ml of 10 mM reduced glutathione solution, pH 7.4. Samples were analyzed by SDS-PAGE and western blotting using an anti-GST antibody (Pharmacia).

2.2 Cloning and expression of murine α -CaMKII in the bacterial expression vector PQE 30/31

2.2.1 Plasmid and Bacterial Strains

Plasmid PQE30 (QIAGEN) (bla/Amp^r) was used to allow a high level expression of 6 $\times$ his-tagged proteins in *E. coli* based on the T5 promoter transcription-translation system. Low copy plasmid pREP-4 (kan^r) was used to regulate the expression of cloned murine α -CaMKII by constitutive expression of lacI^q . *E. coli* M15 [pREP4] based on *E. coli* K12 ($\text{Nal}^S \text{Str}^S \text{rif}^S \text{lac}^- \text{Ara}^- \text{Gal}^- \text{Mtl}^- \text{F}^- \text{RecA}^+ \text{Uvr}^+ \text{Lon}^+$), was used as host strain for electroporation and/or expression. Media used in course of experiments: 20 g/L LB-

agar plates or 20 g/L LB + 15 g/l agar-agar containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin.

2.2.2 Cloning

Complementary DNA of murine α -CaMKII encoding genes were subjected to PCR using engineered primers harboring *Bam*HI and *Sal*I restriction sites (1960B-TAATTGTCGACTTGCGCACTTTGGTGTCTT as forward primer with 925-CGCGTGGATCCCCCATGGCTACCCATCACCT as reverse primer) when cloned into pQE30. PCR amplification of genes was performed according to the previously described protocol for pGEX vector cloning and expression.

PCR products were purified and PCI (phenol chloroform Isoamyl alcohol 24:1) extracted prior to digestion. Fifty µl of 0.5 µg/µl of PCR products, 8 µl of react buffer 6 (Gibco-BRL), 10 units of each *Bam*HI and *Sal*I (Gibco-BRL) were added to 0.5 ml tubes containing 12 µl of ddH₂O. Tubes were incubated at 37°C for 3-4 hrs and PCI extracted day after. The concentration of DNA was determined at OD₂₆₀. Vector pQE30 was also subjected to the same digestion protocol.

The same protocol as the one for pGEX vectors was employed for ligation of PCR products into pQE30. Transformants carrying pPQE30- α -CaMKII and its mutants were selected through Southern colony hybridization, diagnostic 0.6% agarose gel profiling and automated sequencing.

2.2.3 Expression and Purification of pQE30- α -CaMKII constructs

The following protocol was employed in the production of 6×his-tagged α CaMKII and its carboxyl-terminus mutants from standard 100 ml cultures of *E. coli* M15

containing PQE30 plasmids. Cultures inoculated with 5 ml of overnight grown cultures at 37°C were allowed to grow to $OD_{600} \approx 0.6$, incubated with IPTG to a final concentration of 0.5-1 mM, and then incubated for an additional 4-5 hrs. Cells were pelleted at $4000 \times g$ / 4°C (20 min) and stored at -20°C.

Purification under denaturing conditions: Cells were lysed in 5 ml/g wet weight of 8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris-Cl, pH 8.0, mixed gently at 4°C for 30-40 min and centrifuged at $10,000 \times g$ for 20-30 min at 4°C to pellet the cellular debris. One ml of the 50% Ni-NTA slurry was added to 2-5 ml of lysate and mixed gently at 200-250 rpm on a rotary shaker for 30-60 min at 4°C. Lysate-resin mixture was then carefully loaded into a column and washed with 2×4 ml washing buffer (8 M urea, 0.1 M NaH_2PO_4 and 0.01 M Tris-Cl, pH 6.0). Recombinant proteins were eluted with three $\times$ 0.25 ml of elution buffer (pH 4.0). Twenty μ l samples were analyzed by SDS-PAGE.

Purification under native conditions: Cells were lysed in 2-5 ml/g wet weight of 50 mM NaH_2PO_4 , pH 8.0; 300 mM NaCl; 5-10 mM imidazole, mixed gently at 4°C for 30-60 min and centrifuged at $10000 \times g$ for 20-30 min at 4°C to pellet the cellular debris. One ml of the 50% Ni-NTA slurry was added to 2-5 ml of lysate and mixed gently at 200-250 rpm on a rotary shaker for 60 min at 4°C. Lysate-resin mixture was then carefully loaded into a column and washed with 2×4 ml washing buffer (50 mM NaH_2PO_4 , pH 8.0; 300 mM NaCl and 25 mM imidazole). Recombinant proteins were eluted with $3 \times$ 0.25 ml of elution buffer (50 mM NaH_2PO_4 , pH 8.0; 300 mM NaCl and 250 mM imidazole). Twenty μ l of samples was analyzed by SDS-PAGE.

2.3 Cloning and expression of murine α -CaMKII in yeast *Pichia pastoris* 6 $\times$ Histidine tag vectors.

2.3.1 Plasmids, Bacterial and Yeast Strains (Invitrogen, EasySelect™ *Pichia* Expression Kit).

Plasmid pPICZB/ α A was used as a shuttle vector between *E. coli* TOP10F' or DH5 α and the methylotrophic yeast, *Pichia pastoris*, to allow a high level expression of 6 $\times$ his-tagged proteins under tight control of the alcohol oxidase (AOX1) gene promoter, when induced by methanol. Yeast *P. pastoris* strain KM71 Mut^S (Methanol Utilization slow), Arg⁻, His⁻ and GS115 a Mut⁺, His⁻ strain were used as the expression host. Media used in the course of experiments were 15g/L LB-agar YPD/YPDS (1% Yeast extract, 2% Peptone and 2% Dextrose/Sorbitol) media or 15g/L agar-agar containing 100 μ g/ml of Zeocin™ (a member of the bleomycin/phleomycin family of antibiotics). Low salt LB medium (1% tryptone, 0.5% NaCl and 0.5% yeast extract pH 7.5) was used as the medium of choice for transformation. A final concentration of 0.004% histidine was required when dealing with the host strains KM71, GS115 (histidine auxotrophs) transformed with pPICZ or pPICZ α vectors.

2.3.2 Cloning

Murine α -CaMKII encoding cDNAs were subjected to PCR and cloned into shuttle vectors pPICZB/ α A. PCR amplification of genes was performed according to the previously described parameters for cloning and expression in pGEX and/or pQE vectors using primers harboring *Eco*RI and *Xba*I (2619-AAGATGTGCGACCCTGGAATG, 2934-CTGCCGCATTGAGAATTCATC as forward primers and 1519-

ATGGTAGCCATGGGGGATCCA as sole reverse primer). The same ligation protocol as the one used for pGEX vectors was employed. Transformants carrying pPICZB/ α A-CaMKII and mutants were selected by diagnostic 0.6% agarose gel profiling and automated sequencing.

2.3.3 Purification of 6 $\times$ His-tagged murine α -CaMKII and mutant.

Selected genes ($\sim 1 \mu\text{g}/\mu\text{l}$) were cut and linearized with *SacI* and *PCI* extracted as previously described prior to EasyCompTM transformation of *P. pastoris* (see 2.1.2).

2.3.3.1 Preparation of competent cells for EasyCompTM transformation

Ten ml of YPD was inoculated with a single colony of *P. pastoris* KM71 and incubated overnight at 28-30°C in a shaker (250-300 rpm). The overnight grown cells were diluted to $\text{OD}_{600} \approx 0.1-0.2$ and allowed to grow under the same conditions as above to $\text{OD}_{600} \approx 0.6-1.0$ ($\approx$ for 4-5 additional hrs). Cells were then pelleted at $500 \times g$ for 5 min at room temperature and resuspended in 10 ml of solution I (proprietary Sorbitol solution containing ethylene glycol and DMSO) before they were pelleted again under the same conditions as above, resuspended and stored as 50 μl aliquots.

Transformation protocol: Five μg (5 μl) of linearized DNA was added to the 50 μl aliquot of *P. pastoris* KM71 and 1 ml of solution II (proprietary PEG solution) was added to the DNA/cell mixture. The sample was mixed before it was incubated for 1 hr at 30°C in a water bath or an incubator and then heat shocked at 42°C for 10 min. The cells were split into two tubes and 1 ml YPD was added to each, followed by incubation at 30°C for 1 hr. Cells were then pelleted at $3000 \times g$ for 5 min at room temperature. Cells were resuspended in 500 μl of solution III (proprietary Salt solution for washing and plating

transformed cells) and pelleted again under the same conditions as above. Finally, they were resuspended in 100 μ l of solution III and were used to inoculate YPD Zeocin™ plates, which were then incubated at 30°C for 2-4 days.

2.3.3.2 Purification of 6×His-tagged murine α -CaMKII and mutant

The protocol is for a 200 ml standard expression culture of *P. pastoris*: Cells ($OD_{600} \approx 0.5-0.6$) were first harvested by centrifugation at 3000×g and were resuspended in 15-20 ml Breaking Buffer (BB, 50mM sodium phosphate, pH 7.4, 1 mM phenyl-methyl-sulfonyl fluoride (PMSF), 1 mM EDTA and 5% glycerol, with complete™ protease inhibitor cocktail (Boehringer Mannheim). Cells were centrifuged for 10 min at 3000×g at 4°C, resuspended in 50 ml BB, and a half volume of acid washed glass beads was added and mixed for 30 sec. Sonication was repeated 5-10 times while keeping the cells on ice for 30 sec between each step. Samples were pelleted at 4°C for 10 min at 12000×g. The protocol for purification of pQE-6×His-tagged proteins was employed.

2.4 Production of liposomes and binding assay

Multilamellar vesicles (MLVs) were prepared according to the following protocol. In order to produce lipid films with various percent compositions the desired amount of phospholipid (DOPC and/or DOPS) was placed in a scintillation vial and enough chloroform/methanol (2/1 v/v) was added to give a final phospholipid concentration of 25 mg/ml. Mixtures with the lipid compositions 100% PC, 50/50% DOPC/DOPS, and 80/20% DOPC/DOPS (Avanti) were dried down, using nitrogen gas, placed under vacuum for 1 hr and sealed with parafilm and stored at -20°C. The lipid film was hydrated in buffer A (50 mM KCl, 10-20 mM HEPES, 1 mM EDTA, pH 7.4),

freeze-thawed 5 times using liquid nitrogen and a 37°C water bath with mixing between each freezing cycle. A phosphate assay was performed to determine lipid concentration ($100\text{PO}_4=5\mu\text{l} \times 100\text{nmol/A}_{820}$). Large unilamellar vesicles were prepared using a Liposofast pneumatic (Emulsiflex® Avestin, Inc.). Lipids were extruded 10-11 times through two polycarbonate membranes. (100 nm pore diameter, Avestin Inc.).

2.4.1 Membrane Binding assay

Liposomes, murine α -CaMKII and/or mutants and buffer B (20 mM HEPES, 1 mM EDTA, 50 mM KCl, pH 7.4, 1 mg/ml BSA) were brought to final volume of 50 μl in an eppendorf tube. The sample was briefly mixed and then incubated on ice for 15 min before it was subjected to centrifugation at 15000 $\times$ g rpm for 30 min at 4°C. The top 40 μl of solution (supernatant) was transferred to another eppendorf tube and 10 μl of 10% Triton X-100 was added. The pellet was resuspended with 30 μl of buffer B and 10 μl 10% Triton X-100. Quantitative dot immunoblotting was performed on both pellet and supernatant to determine bound protein (see 2.5.4).

2.5 Miscellaneous techniques

2.5.1 SDS-PAGE

The recombinant proteins were anticipated to have molecular weight of 40-70 kDa and therefore 10-12% (mid-range) SDS-PAGE gels were used. To prepare two 12% separation gels, 12 ml of acrylamide/bis-acrylamide (37.5/0.5), 20 ml of 2 $\times$ separation buffer (0.75M Trizma, 0.2% SDS, pH 8.6), and 8 ml ddH₂O were added into a 50 ml beaker and mixed before 25 μl of N, N, N', N'-tetramethyl-ethylenediamine (TEMED)

and 110 μ l of 10% ammonium persulfate (APS) was added. The solution was poured into mini-PROTEAN II gel molds (BIO-RAD) and allowed to polymerize. In order to produce a smooth edge, drops of isobutanol/water (1:2) was added on the top of gels.

To prepare two 3.75% stacking gels, 0.75 ml of acrylamide/bis-acrylamide (37.5/0.5), 4 ml of 2 $\times$ stacking buffer (0.25M Trizma, 0.2% SDS, pH 6.8), and 3.25 ml ddH₂O were added into a 25 ml beaker and mixed before 8 μ l of TEMED and 24 μ l of 10% APS was added. The proper comb was placed and the stacking gel was poured avoiding formation of bubbles after isobutanol/water (1:2) was removed from top of the solidified separation gels. One of the gels was used for western blotting and the other for SDS-PAGE analysis. Twenty μ l of sample recombinant proteins and 2 μ l of 1 $\times$ sample buffer (0.2 M Tris-buffer and 2% glycerol) was mixed in a 1.5 ml eppendorf tube and allowed to boil for 2 min before samples were transferred to wells. The gels were run at 90-120 volts for about 120 min.

2.5.2 Western blotting

The proteins were electrophoretically transferred (BioRad, 0.3A, 12-15 hrs) to nitrocellulose paper (Optitran), where they were bound irreversibly. The proteins on the membrane were stained with 5 ml of Ponceau Red 0.5%, 3% TCA. (Sigma) for 5 min with gentle shaking, rinsed quickly with ddH₂O. The proteins were fixed in 10% acetic acid (v/v), 25% isopropyl alcohol (v/v), for 15 min and rinsed 3 times with ddH₂O. It was blocked with blocking solution (25 g fat free skimmed milk/L in 1 $\times$ Tris-buffered saline containing 0.5% Tween-20) for 60 min with gentle shaking. The primary rabbit polyclonal IgG antibody (G541, affinity pure, Santa Cruz Biotechnology, 100 μ g/ml, 500 $\times$ dilution) was added and incubated for 90 min. The nitrocellulose membrane was

washed 4 times (2 x 1 minute and 2 x 15 min) with blocking solution, incubated with secondary antibody (goat anti-rabbit horse radish peroxidase, HRP, 1/10000 dilution) for 90 min in 1x Tris-buffered saline containing 0.5% Tween-20 (no dry milk). The nitrocellulose was washed 4 times (2 x 1 minute and 2 x 15 min) with the same buffer. An additional washing (2 x 5 min) was done with the same buffer, without Tween-20. The paper was air dried, wrapped with plastic wrap and kept at 4 °C until Enhanced Chemiluminescent (ECL) analysis.

2.5.3 Protein quantification (BCA protein assay)

A standard curve was prepared using BSA (Pierce albumin standard 1mg/ml in ddH₂O) and ddH₂O, to the final volume of 100 µl (10-15 µl of unknown protein was used). A working solution was prepared by mixing 50 parts [Bradford Coomassie Assay (BCA) reagent A (Pierce)] with 1 part Reagent B (BSA protein assay reagent B, Pierce). Two ml of working solution was added to unknowns and standards followed by a brief vortex. The solutions were incubated at 37 °C for 30 min, then were cooled to room temperature, and the absorbances at 562 nm were measured (in a DU-65 spectrophotometer Beckman Instruments, Inc) against ddH₂O. The concentrations of unknown proteins were calculated using linear regression of standards and unknowns.

2.5.4 Enhanced ChemiLuminescent (ECL) analysis

ECL was performed in the dark room in the presence of Kodak Utility Voltage Lamp. The nitrocellulose membrane containing the desired proteins was soaked in a mixture of 5 ml Lumionl/Enhancer Solution and 5 ml Stable Peroxide Solution (Proprietary solution) Super Signal West Pico (Biolynx Inc), for 3 min with gentle

shaking. The nitrocellulose was air-dried, wrapped in plastic wrap and exposed to Kodak scientific imaging film (Kodak BioMax) in a spectroline cassette (Harmer) for 30-60 sec. The film was placed in the developing solution (Kodak GBX developer and replenisher) for 3 min, rinsed with tap water and fixed in fixation solution (Kodak GBX fixer and replenisher) for 3 min.

2.5.5 PCR fragment Isolation and purification

About 150 µl of Direct Purification Buffer (50 mM KCl, 10 Mm Tris-HCl, pH 8.8, 1.5 mM MgCl₂, 0.1% Triton x-100) and up to 300 µl of PCR products were mixed 3 times within one minute. One ml of PCR Preps DNA Purification Resin, (Promega), was added to the mixture, mixed gently, and was subjected to vacuum (Promega). It was washed with 2 ml of 80% isopropanol, was centrifuged (Eppendorf centrifuge 5415, 2 min to withdraw all the alcohol. DNA was resuspended in 50 µl of TE buffer (10 mM Tris-HCl, pH 7.5, 1mM EDTA) and stored at -20 °C.

Figure 2.1 Directional PCR Cloning

Scheme of directional PCR experiments to produce C-terminal deletion mutants of α -CaMKII. The PCR fragments with engineered restriction sites (e.g. *Bam*HI/ *Eco*RI for GST-CaMKII, *Bam*HI/*Sal*I for bacterial 6 $\times$ his-CaMKII, and *Eco*RI/*Xba*I for yeast 6 $\times$ his-CaMKII mutants) were digested with appropriate restriction enzymes. Diagram shows the digested products were ligated into pGEX (2T/4T1) plasmids used in production of GST-fusion proteins. Other PCR fragments were also produced in the same manner and cloned into other vectors namely pQE-30 or pPICZ to produce 6 $\times$ his tagged deletion and full-length peptides in bacteria and yeast respectively (not included in diagram).

Clones Were Transferred Into Bacteria And Were
Expressed. Proteins Were Purified And Used In
Liposome Binding Assays.

Chapter 3.

Results

3. RESULTS

3.1 Cloning and expression of murine α -CaMKII using bacterial GST fusion vectors pGEX2T/4T1

The PCR products (~1Kb) were purified (Wizard™ PCR purification system, Promega) after being subjected to restriction digestion to insure better ligation results (Figure 3.1). Plasmids were maintained in *E. coli* DH5 α /JM103, to prevent dimerization/trimerization of DNA upon storage at 20°C.

The GST fusion system (pGEX2T) was the first choice for cloning and expression of α -CaMKII cDNA encoding 50-60 kDa proteins in a prokaryotic system. The forward primers were 5'-labeled with ^{32}P and used in the Southern colony hybridization (SCH) experiments (Figure 3.2). The SCH results given in Figure 3.2 (b, c) are examples of those corresponding to the shortest and the longest C-terminal deletion mutants (pGEX-1960 and pGEX-2200) using pGEX system. These results show the advantage of screening positive clones over DNA mini-prep analysis. Hundreds of positive transformants were checked for the desired plasmids. DNA mini-prep analysis was performed to confirm cloning. The results of SCH experiments, if desired, can give an estimate of success of cloning and/or transformation. For example, the ratio of signaling: non-signaling clones was determined for pGEX-1960 and pGEX-2200 C-terminal deletion mutants of α -CaMKII cDNA, and the efficiency of cloning was around 80-90%. Examples of successful cloning are shown in figure 3.3.

Protein expression and purification, on the other hand, brought about many problems and many trials were needed to improve the purification protocol for GST fused

proteins. In order to produce larger amount of protein, a column method was used and the cultures were scaled up. In addition, cells were resuspended in lower volumes of lysis solutions to increase the concentration of recombinant proteins in the clear lysate before purification.

Recombinant GST fusion proteins appeared as multiple bands (30-45kDa). The total yield of recombinant proteins after induction was high showing that translation of the encoded gene was initiated and continued successfully long after commencing induction (Figure 3.4). Full-length α -CaMKII was also fragmented but the amount of undesired fragments was negligible (Figure 3.4 A, lane 3). In addition, full-length α -CaMKII was resolved only on 8-10% SDS-PAGE gels and did not appear as multiple bands on gels of high acrylamide concentrations (12-16%). This suggests that the two-banded pattern may be caused by two different denatured isoforms of α -CaMKII (Figure 3.4 A and C). SDS-PAGE profiles (Figures 3.4 B and C) show that multiple band formation of recombinant murine α -CaMKII was continually evident during induction and therefore was not a result of purification.

In an attempt to reduce the number of multiple bands, several techniques were tried and many additional parameters were incorporated in the expression and purification steps such as alternative salt concentration, storage and purification conditions. *E. coli* BL21 (DE3) a protease-deficient strain *F*⁻, *ompT*⁻, *hsdS* (*rB*⁻, *mB*⁻), (Pharmacia) was used to promote higher production of recombinant proteins. A protease inhibitor cocktail (Complete™ Boehringer Mannheim), containing a variety of inhibitors was added to all purification solutions. Protease inhibitor phenyl-methyl-sulfonyl fluoride (PMSF) was also used at low concentrations (0.1 mM) but the pattern of degradation

continued to appear in SDS-PAGE and western blots of eluates. Milder conditions of lysis, use of B-PER (Pierce) bacterial solution with lysozyme and/or shorter periods of sonication, did not produce pronounced effects in terms of multiple band formation (compare Figures 3.4A and 3.5A).

Purified proteins stored at -20°C were found to be susceptible to multiple band formation. Therefore, it is recommended that protein is purified just before performing a bioassay to insure full length protein.

Dialysis of peptides is necessary, as the high ionic strength of elution solutions may prove problematic when performing membrane-binding assays. Dialysis of recombinant proteins had a negligible effect on them in terms of degradation.

3.2 Cloning and expression of murine α -CaMKII in pQE system

E. coli JM103 was used to replace the usual host *E. coli* M15 [pREP4] as it has the lac^{lq} sequence and T7 promoter needed to keep the system under tight repression before induction (Figure 3.6). This makes the cloning of foreign cDNA in the pQE system much easier since a double plasmid system for tight repression is not needed.

During expression of the pQE mutant α -CaMKII (analog of α -CaMKII-1960, the shortest mutant in pGEX system), the SDS-PAGE profiles first came out as smears (Figure 3.7A). The problem was fixed by increasing the salt concentration in solutions when performing purification under native conditions (Figure 3.7B). Purification under denaturing conditions produced a higher yield than that using native conditions (70-80% of total protein in cell lysates compared to 40-50%; Figures 3.7B and C). The time course of induction profile in Figure 3.8 and its western blot show multiple banding occurred

before purification (Figure 3.4). Both α -CaMKII and its mutants produced using this system were used as benchmark samples in binding assays.

3.3 Cloning and expression murine α -CaMKII in pPICZ system (yeast vector)

To improve the production of α -CaMKII and one of its mutants, the yeast vector system, pPICZ was used. The cloning and maintenance of genes in *E. coli* TOP10F' was as easy and efficient as those done in the pQE and pGEX prokaryotic systems (Figure 3.9). The shuttle constructs were transferred to the yeast host efficiently but selection of the positive clones was not efficient when the protocol provided by Invitrogen Inc. was followed. Diagnostic PCR of the genomic DNA to assure integration of the insert was time and money consuming with little result.

The pPICZ α constructs did not produce any detectable proteins at all whereas pPICZB constructs again produced just a small amount of degraded peptides (Figure 3.10). Because the quality of proteins in the pGEX and/or pQE system was higher than that of this system, production of recombinant proteins using yeast was abandoned.

3.4 Binding assays

The immuno-binding assay used was designed according to the recombinant protein under investigation. Anti-His antibody was used if recombinant protein was a 6 $\times$ his-tagged protein while anti-GST was used as primary antibody against GST recombinant proteins including bovine Synapsin Ia (positive control, purified from bovine brain). None of the recombinant α -CaMKII showed any binding to the liposomes tested. The concentration of peptides was high enough to produce detectable results (2-5 mg/ml) (Figure 3.11). Bovine Synapsin Ia (positive control which binds to vesicle

membranes) was used as a positive control for binding. Comparison of supernatant to pellet fractions from binding assays showed that synapsin Ia displayed differential binding to membranes with different lipid compositions (100% DOPC, 80:20% ratio, and 50:50% ratio DOPC: DOPS) (Figure 3.11). Recombinant murine α -CaMKII and mutants (6 $\times$ his-tagged proteins and/or GST fusion proteins) did not bind to membranes at concentrations between 2-5 mg/ml protein.

Figure 3.1. PCR amplification of murine α -CaMKII C-terminal mutants encoding genes.

Lanes 1-4 correspond to PCR products before the Wizard™ (Promega Inc.) extraction used in cloning of α -CaMKII cDNAs into pGEX (2T/4T1) plasmids. Lanes 1 is Δ C α -CaMKII-1960, lane 2 is Δ C α -CaMKII-2043, lane 3 is Δ C α -CaMKII-2126, and lane 4, corresponds to Δ C α -CaMKII-2200. Lane 1 (Δ C α -CaMKII-1960) cDNA was cloned to produce the shortest of deletion mutants where lane 4 cDNA was used in production of longest of mutants. Δ C α -CaMKII-2043 and 2126 were intermediate in length compared the Δ C α -CaMKII-1960 and 2200 and were used for expression of intermediate proteins. Lanes 5 to 8 show the same PCR products after extraction. 1 Kb DNA ladder is shown in lane 9. Complementary DNAs were engineered to have *Bam*HI and *Eco*RI restriction sites at 5' and 3' respectively.

Figure 3.2. Typical cloning procedure

A. pGEX2T vector was cut with individual and both enzymes (*Bam*HI and *Eco*RI). Lane 1 is digested with *Bam*HI. lane 2 is digested with *Eco*RI, lane 3 is double digested with *Bam*HI and *Eco*RI and lane 4 is undigested pGEX2T vector. PGEX2T- α -CaMKII (used as template for PCR) was also digested with same enzymes. Lane 5 is double digested, lane 6 is digested with *Bam*HI, lane 7 is digested with *Eco*RI and lane 8 corresponds to undigested PGEX2T- α -CaMKII. B. Southern colony hybridization Profiles of CaMKII-1960 (shortest) and C. CaMKII-2200 (longest) mutants as examples for SCH of *E. coli* JM103 transformed with ligation products. Positive clones were selected and checked for the expected plasmid sizes.

Figure 3.3. Diagnostic restriction analysis.

Plasmids pGEX Δ C α -CaMKII-1960, 2043, 2126, 2200 (lanes 1-4) were cut with *Bam*HI and *Eco*RI and run against Δ C α -CaMKII (lane 5) and a 1 Kb DNA ladder (lane 6). Top arrow points to linearized vector (~4900 bp) and the bottom arrow correspond to inserts of 1018 to 1636 bp.

Figure 3.4. Purification of GST mutants.

A. Purification of GST-CaMKII-1960, 2043, 2126, 2200 mutants (lanes 7 to 10, respectively, represented by top arrow ~75 to 50 kDa) from 100 ml culture of *E. coli* BL21 (10% SDS-PAGE). Lane 1 shows high molecular protein markers. Lane 3 is full length GST-CaMKII. Lanes 4, 5 and 6 correspond to steps of purification (flow through, first and second washings) respectively. **B.** western blots which show the multiple bands and their increase during induction. **C.** 12% SDS-PAGE profile of time course of induction. The multiple band pattern is clear in samples of crude cell lysates even before purification where arrows point to the products.

Figure 3.5. Purification steps of full-length murine α -CaMKII from pGEX2T- α -CaMKII plasmid.

A. HMW represents high molecular weight markers. F is flow-through, S is soluble protein. W1, W2 and W3 are washings 1, 2 and 3. E1 and E2 are eluates 1 and 2. **B.** Purified GST- α -CaMKII. Lane 1 shows high molecular weight markers and Lane 2 shows the double-banded protein. **C.** Western blot of protein obtained using anti-GST antibody. Arrows show multiple banding patterns in the purified protein.

Figure 3.6. Construction of cloned α -CaMKII into pQE30 plasmid (Qiagen).

A. Murine α -CaMKII encoding cDNAs were subjected to PCR using engineered primers, harboring *Bam*HI and *Sal*I restriction sites, and cloned into pQE30. PCR amplification of cDNAs was performed according to a previously described protocol for pGEX vector cloning and expression (see page 13-15). Lane 1 shows a 1 Kb ladder. Lane 2 is the PCR product of murine α -CaMKII (~1400 bp). **B.** 0.6% agarose gel electrophoresis profile showing screened mutant of α -CaMKII. Lane 5 is a positive clone versus a negative clone (pQE-30 in lane 4) and supercoiled DNA is shown in lane 3. **C.** The clone in lane 7 (4500 bp) is shown versus supercoiled DNA ladder in lane 6

Figure 3.7. Purification of 6×his-tagged peptides.

- A. Production of 6×his-tagged murine α -CaMKII C-terminal mutant from standard 100-ml cultures of *E. coli* M15 containing PQE30 vectors carrying appropriate cDNA.
- B. Lane 3 represents the two banded protein against low and high molecular weight markers (lanes 1 and 2, respectively). C. Lane 4 is purified 6×his-tagged α -CaMKII (one band only).

Figure 3.8. Time Course of Induction.

A. Time course of induction of mutant α -CaMKII (0, 1 and 2 hrs after induction). The pattern shows multiple bands occurred before purification. B. Western blot of the double-banded mutant α -CaMKII. purified using the 6 $\times$ his-tagged pQE system shows appearance of multiple banding pattern right after starting induction.

Figure 3.9. PCR Fragments for Cloning in yeast Vectors.

A. PCR amplification of murine α -CaMKII cDNA. α -CaMKII encoding cDNA was subjected to PCR using engineered primers (with *Eco*RI and *Xba*I sites) into shuttle vectors pPICZB/ α A (Invitrogen). PCR amplification of cDNAs, was performed according to the previously described parameters in the protocol for cloning and expression in pGEX and/or pQE vectors. Lanes 2 and 3 correspond to mutant and 6 $\times$ his-tagged -murine α -CaMKII encoding cDNAs against linear 1 Kb ladder in lane 1. **B.** Lane 5 is pPICZ vector, lanes 6 (mutant 6 $\times$ his-tagged) and 7 are positive (6 $\times$ his-tagged-CaMKII) clones in a DNA size screening experiment. Lane 4 is the 1Kb ladder.

Figure 3.10. Purification of-CaMKII and Mutant Using Yeast Vector.

Purification of pPICZB/ α A-6 $\times$ His-tagged α -CaMKII. Lane 1 is the standard double-banded protein purified in a prokaryotic system (6 $\times$ His-tagged α -CaMKII). Lane 2, 3, and 4 show multiple bands in proteins purified from yeast *P. pastoris* KM71. Lanes 5 and 6 are before and after induction, respectively.

Figure 3.11. Liposome binding assays.

A. Immuno-dot blots using bovine synapsin Ia (positive control which binds to vesicle membranes) to test affinity of recombinant 6×his-tagged/GST α -CaMKII to membranes. Comparison of supernatant (lane 1) to pellet (lane 2) fractions from a binding assay shows how bovine synapsin Ia binds (positive control) to membranes at different liposome compositions (100% DOPC and 50:50% DOPC: DOPS). **B.** Recombinant murine α -CaMKII and mutants (6×his-tagged proteins and/or GST fusion proteins) did not bind to membranes at concentrations between 2-5 mg/ml protein (Lane 1 and Lane 2 correspond to supernatant and pellet fractions respectively). **C.** Anti-GST immuno-dot blot of GST-CaMKII as positive control.

Chapter 4.

Discussion

4. DISCUSSION

α -CaMKII is a calcium-dependent protein kinase, which is associated with synaptic vesicles. In response to elevated calcium levels, it phosphorylates synapsin I leading to dissociation of synaptic vesicles from the cytoskeleton, thereby allowing them to bind to presynaptic membranes for fusion and neurotransmitter release.

Murine α -CaMKII and C-terminal deletion mutants were expressed in both prokaryotic and lower eukaryotic systems to investigate the affinity of recombinant α -CaMKII for membranes. The production of murine α -CaMKII in prokaryotic and/or lower eukaryotic systems involved a great deal of trouble shooting. Therefore, it is imperative to first discuss the cloning and expression of the recombinant proteins.

4.1 Cloning and expression of murine α -CaMKII using bacterial GST (Glutathione S Transferase) fusion vectors pGEX2T/4T1

The purified GST- α -CaMKII fusion protein and mutants appeared as multiple bands in SDS-PAGE and western blot profiles. The time course of induction showed that multiple banding patterns occurred during the log phase of growth before harvesting the cells. The multiple bands may be due to proteolysis of the fusion protein. Another possible explanation for the multiple bands may be premature termination of translation producing proteins of smaller size. The banding pattern may be a result of the combined effects of the above-mentioned possibilities. The pGEX4T-1 clones also produced the same pattern of multiple protein bands when expressed in *E. coli* BL21.

4.2 Cloning and expression of murine α -CaMKII in bacterial N-terminal

6×histidine tag vector PQE30/31.

Cloning and expression of the murine α -CaMKII cDNA and its C-terminal mutants in the pQE30 (type IV) system was tried as a way to avoid multiple protein bands. However, expression of 6×his-tagged proteins also encountered some problems. The recombinant proteins produced smears when run on SDS-PAGE in the first trials but the problem was solved by increasing the ionic strength of the purification solutions. The SDS-PAGE pattern of proteins yielded two bands and their yields were at least three-fold higher, compared with the yield of GST fusion mutants.

The results from studies of the time course of induction on the 6×his-tagged mutant and the full length α -CaMKII in cell lysates were consistent with the appearance of multiple bands during induction implying that multiple bands were not the result of protein shearing during purification. In addition, protein production from other cDNAs (e.g. prokaryotic nitroreductase genes and short domains of synapsin I) cloned and expressed in the same system produced one-banded proteins with high yield. The results suggest that α -CaMKII is subject to degradation during induction.

4.3 Cloning and expression of murine α -CaMKII in yeast *Pichia pastoris*

6×Histidine tag vectors.

Cloning and purification of 6×his-tagged proteins was tried in a lower eukaryotic system (yeast *P. pastoris*). Production of murine α -CaMKII in this system proved to have no advantage over prokaryotic systems. The system involved high shearing techniques to

break the yeast cell wall, which generated heat; this is a possible reason for the SDS-PAGE degradation pattern seen (Figure 3.10).

4.4 Binding Assays

Murine α -CaMKII consists of a small C-terminal β -sheet domain (association domain in the case of α -CaMKII) and a larger α -helical domain encompassing most of the enzyme. Functionally important parts of the enzyme such as substrate and ATP binding sites and the autoinhibitory sequence are located in the cavity produced in between the two lobes (Cruzalegui *et al.*, 1993). Direct binding and/or association between the synaptic vesicle membrane and the C-terminus of α -CaMKII may rely on an interaction between positively charged amino acid residues of the β -sheet domain with negatively charged phospholipid head groups as occurs for several other extrinsic proteins (e.g. cytochrome C and spectrin). Murine α -CaMKII also has a phenylalanine-rich region (residues 370-390) that could be involved in binding to lipid vesicles.

A synaptic vesicle-associated form of CaMKII can be purified directly from rat brain and is insensitive to dissociation by increasing ionic strength (Benfenati *et al.*, 1996). However, recombinant α -CaMKII, regardless of the fusion tags (GST and/or 6 $\times$ his-tagged), did not show any affinity for the liposomes tested.

Many factors might have played roles in unsuccessful binding assays. The 26-kDa fusion partner, GST, may easily disrupt and/or weaken direct associations with phospholipid head groups. The GST was not removed from our peptides since it was needed to detect the proteins via immunological assays using anti-GST antibody. The

only commercial antibody against α -CaMKII recognizes epitope at C-terminal, which was deleted in our mutant proteins.

The postsynaptic form of the enzyme is a soluble oligomer holoenzyme that associates with NMDA channels and phosphorylates AMPA channels to enhance their responsiveness to glutamate release. This, and the fact that the enzyme co-purified with SVs is insensitive to high ionic strength, may actually be a hint that the enzyme may have an indirect form of association with membranes. The enzyme may well rely on interaction with other proteins to bind to vesicles, such as α KAP, an anchoring protein (in α -CaMKII isoform from rat muscle), found by Bayer *et al.* (1998). The molecular crowding at the regions of action of presynaptic α -CaMKII may also be important in explaining the localization of enzyme in the reserve pool of synaptic vesicles for an enzyme otherwise soluble at the postsynaptic membrane.

In skeletal muscle, an alternative non-kinase translation product, α -KAP (α -CaMKII association protein), is also expressed from the α -CaMKII gene. α -KAP consists of a C-terminal region that is identical to the association domain of alpha CaMKII, with the exception of 11 amino acids inserted in the variable region (Bayer *et al.* 1998). The N-terminal sequence of α -KAP is very hydrophobic and not present in any known CaMKII protein. The catalytic and regulatory domains of α -CaMKII are missing in α -KAP. α -KAP may function as an anchoring protein for CaMKII on synaptic vesicles.

4.5 Further experiments and recommendations

Using mammalian systems for expression of α -CaMKII cDNAs is recommended because full-length recombinant proteins may be easier to obtain. Devising a more reliable and quantitative method for measuring liposome binding would also help to make experiments much more reliable. The immuno-binding method used in this study may not have been sensitive enough, at least in the case of recombinant murine α -CaMKII and its C-terminal mutants. Producing smaller fragments through directional cloning may be a better way to investigate the membrane association of α -CaMKII in neurons. Generating antibodies against the recombinant proteins themselves may allow different actions of mutants to be followed directly, not relying on their affinity tags, which may well be responsible for the different behavior of the recombinant proteins towards membranes. In addition, including α KAP, actin and/or other proteins in the binding reaction will more closely simulate the environment of the presynaptic nerve terminal.

4.6 Conclusions

Murine GST- α -CaMKII was expressed in *E. coli* and purified using glutathione affinity resin. SDS-PAGE and western blot analyses of recombinant full-length murine GST- α -CaMKII showed multiple bands indicating that smaller molecular weight versions of the protein were binding to the glutathione beads. This was most likely due to proteolysis of the fusion protein, but may also have been caused by premature termination of translation. Recombinant murine His₆- α -CaMKII and C-terminal deletion mutants were also expressed in bacteria, and only two bands were detected in SDS-PAGE and western blot analyses. These fusion proteins were used in liposome binding assays.

Expression of murine 6× His₆-α-CaMKII and its C-terminal mutants in *P. pastoris* was attempted but the yield of recombinant protein was too low to be used in liposome binding assays.

Liposome binding assays did not show any binding of full-length His₆-α-CaMKII or His₆-α-CaMKII deletion mutants to the liposomes tested. These results suggest that the synaptic vesicle-associated form of α-CaMKII is anchored to the vesicle membrane through a mechanism other than direct binding to membrane phospholipids.

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