CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II ANCHORING TO L-TYPE CA^{2+} CHANNELS BY THE BETA SUBUNITS ENHANCES REGULATORY PHOSPHORYLATION OF THE BETA SUBUNIT AT THR498

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CHAPTER I

INTRODUCTION

Survival of all living organisms in the environment requires the ability to sense and respond to countless, ever-changing types of stimuli. Ca^{2+} directly or indirectly supports all essential biological processes involved in the adaptation of cells to changes in both their internal and external environment. The increases in cytosolic Ca^{2+} necessary to trigger physiological processes result from Ca^{2+} mobilization by a plethora of Ca^{2+} cycling proteins at the plasma membrane or intracellular stores. This thesis explores biochemical mechanisms underlying regulation of Ca^{2+} entry through L-type voltage-activated channels by Ca^{2+}/Calmodulin-dependent kinase II (CaMKII) in neurons and cardiomyocytes.

1.1 COMMON ROLES OF CA^{2+}

This section discusses selected roles of Ca^{2+} in the birth, life and death of cells and organisms.

Developmental roles of Ca^{2+}: The subject has been more extensively reviewed elsewhere (for example in [1]). Briefly, in mammals, Ca^{2+} triggers fertilization [2, 3], influences cell body polarity and pattern formation in the zygote, plays a role in the proliferation and differentiation of cells into specific types to form tissues and organs. For example, Ca^{2+} regulates the migration and branching of

neurons, and thereby controls the formation of the initial connections within the complex circuitry in the developing brain. The initiation of apoptosis, or controlled death of cells, is important for sculpting, maintaining and renewing the architecture of multicellular organisms and critically dependent on the magnitude and location of Ca^{2+} changes[2, 4]. Thus, Ca^{2+} is critical for normal development of organisms.

Muscle Contraction: Activation of voltage-gated Ca^{2+} channels (VGCC) by membrane depolarization stimulates skeletal muscle contraction[5, 6]. Interestingly, the initiation of Ca^{2+} release from the sarcoplasmic reticulum (SR) by VGCCs, but not entry of extracellular Ca^{2+}, appears be required for skeletal muscle contraction. Ca^{2+} binds to troponin C, stimulating the ATP hydrolysis that powers the movements of myosin and actin during muscle contraction. Cardiac muscle contraction is initiated when extracellular Ca^{2+} enters cardiomyocytes, primarily through voltage-gated Ca^{2+} channels[7, 8], and induces Ca^{2+} release from the SR. The resulting spatiotemporal changes in Ca^{2+} concentration drive acute processes such as the strength and frequency of heart contraction and long-term events such as gene expression[5, 7]. Regulation of Ca^{2+} influx via VGCCs is therefore a central mechanism for controlling heart function.

Neuronal communication: Ca^{2+} is important in regulation of neuronal excitability, transmission of nerve impulses and activation neuronal process that underlie learning and memory[2, 9]. Ca^{2+} channels at the membranes of neurons can

determine not only spontaneous neuronal activity but also their responses to stimulation. In addition, Ca^{2+} is a trigger for neurotransmitter release that is essential for transmission of impulses at chemical synapses. The efficacy of synaptic transmission can be altered, in a Ca^{2+}-dependent manner, by certain intensities and patterns of stimulation. Enhancements and reductions of synaptic transmission efficacy are the hallmarks of long-term potentiation (LTP) and long-term depression (LTD) respectively, though other forms of potentiation and depression also exist. These changes in electrical transmission, along with structural changes in neurons, are commonly referred to as plasticity and are thought to underlie learning and memory. The effects of Ca^{2+} on neurotransmission can be mediated by changes in a plethora of processes, including the synthesis and release of neurotransmitters, the abundance or modification of synaptic proteins, and changes in neuronal cytoskeleton. Some of the functions of Ca^{2+} at neuronal synapses are also important in nonneuronal cells. For example, Ca^{2+} controls secretion of glutamate by photoreceptors, secretion of epinephrine by adrenal chromaffin cells or release of insulin by β cells of the pancreas [4, 10-12].

Pathophysiological roles of Ca^{2+}: In general, a moderate level of Ca^{2+} is required for survival. But extremes of Ca^{2+} concentration trigger apoptosis. For example, elevated Ca^{2+} influx is known to induce cell death, while depletion of Ca^{2+} from the ER or its accumulation in the mitochondria can also induce proapoptotic stress signals. Another form of cell death, necrosis, is less controlled and may be

induced by disintegration of cells by Ca^{2+}-activated calpains. Some of the diseases commonly linked to abherrant Ca^{2+} signaling include cardiac arrhythmia, congestive heart failure, muscular dystrophy [5], Parkinson's Disease[13, 14], epilepsy[15], Alzheimer's Disease[16], ischemic damage[15], and Timothy Syndrome[17]. Abnormal signaling of cell proliferation and growth by Ca^{2+} has been implicated in some forms of cancer[2]. It is therefore critical that Ca^{2+} concentrations are precisely controlled to enable normal bodily functions to occur with minimal risk of injury or death.

1.2 GENERAL ASPECTS CA^{2+} HOMEOSTASIS

The well-orchestrated changes in Ca^{2+} required for physiological signaling rely on separation of intracellular and extracellular pools of Ca^{2+}, and tight control and compartmentalization of Ca^{2+} in cells[7, 9]. Cells have evolved tools or Ca^{2+} cycling proteins to confine Ca^{2+} in the extracellular space and in intracellular stores, and to allow controlled Ca^{2+} influx in response to stimuli (**Figure 1**). The influxes are mostly brief (~1 sec) and localized; rarely is global cellular Ca^{2+} elevated for extended periods.

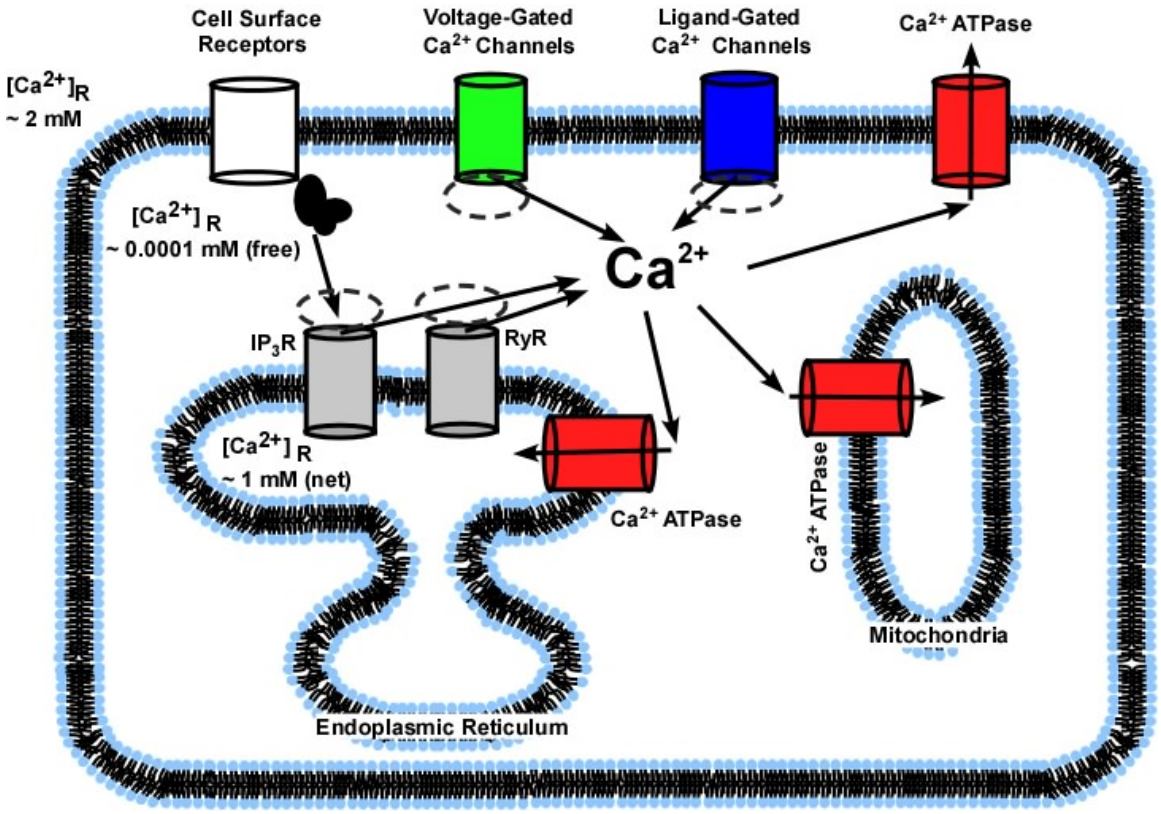

Figure 1. General aspects of cellular Ca2+ homeostasis.

At resting conditions, Ca^{2+} is kept in the extracellular space and in intracellular stores by Ca^{2+}-impermeable membranes and various pumps. Stimulation of cells activates ligand or voltage-gated receptors and channels localized in the plasma membrane or in the membranes of intracellular stores. The resulting increases in cytosolic Ca^{2+} uniquely signal a variety of biochemical and physiological changes in the cell, and ultimately affect the organism. In order to prevent cellular damage, excessive Ca^{2+} entry is prevented by feedback inhibition of receptors and channels, active pumping of Ca^{2+} into extracellular space and intracellular stores, and by Ca^{2+} buffering proteins.

Maintenance of Basal Ca^{2+}: Basal Ca^{2+} levels are kept low (~100 nM) as Ca^{2+} is continuously pumped out of the cytosol into the extracellular space by a plasma membrane Ca^{2+} ATPase (PMCA) and sodium Ca^{2+} exchanger located at the plasma membrane (**Figure 1**). Ca^{2+} is also returned into the ER by the Sarco-Endoplasmic Reticulum Ca^{2+} ATPase(SERCA) and into mitochondria by a Sodium/Ca^{2+} exchanger. The function of these proteins is regulated by prevailing concentrations of Ca^{2+} in the cytosol and inside the Ca^{2+} stores. Fast-acting Ca^{2+}-buffering proteins such as parvalbumin and calbindin also contribute to the maintenance of low cytosolic Ca^{2+} levels[1].

Generation of Ca^{2+} Signals: When cells are stimulated, for example by synaptic activity, Ca^{2+} levels increase, by up to a thousand-fold, in the immediate vicinity of the channels[18]. The electromotive force for the rapid increase in cytosolic Ca^{2+} is a ten thousand-fold gradient of Ca^{2+} concentration across the plasma membrane. Ca^{2+}-levels in the ER and mitochondria are also several orders of magnitude higher than those in the cytosol. Ca^{2+} enters into cells due to activation of receptors and channels located both at the cell surface and on membranes of intracellular storage organelles such as the ER and mitochondria. G-protein coupled receptors (GPCRs) such as mGluR1 and bradykin receptors stimulate phospholipase C (PLC) β resulting in production of inositol (1,4,5) triphosphate (IP3). The resulting activation of IP3 receptors on the ER membrane stimulates Ca^{2+} flux into the cytosol. IP3 production can also be stimulated via activation PLCδ and PLCε by receptor tyrosine kinases and the Ras pathway.

Another well-known route of Ca^{2+} entry from the ER is the ryanodine receptor (RYR). RYR is endogenously activated by cyclic ADP ribose, but its activation by Ca^{2+} is better known due to the Ca^{2+}-induced Ca^{2+} release phenomenon[19].

Even though Ca^{2+} from intracellular stores controls important cellular processes, cells do not make Ca^{2+}; Ca^{2+} must initially enter from the extracellular space. Voltage-gated Ca^{2+} channels (VGCCs) located at the plasma membrane are the main portals of extracellular entry in many cell types[20]. Indeed, so prominent is their role that VGCC activation can reduce Ca^{2+} concentrations in the restricted extracellular space surrounding neurons in intact nervous tissue by a substantial 0.5 mM (~30%)[21] . However, the cytosolic Ca^{2+} concentration changes are transient because most of the Ca^{2+} that enters into the cytosol is rapidly bound by buffer proteins or pumped out of the cell or into intracellular stores.

The mechanisms that elevate and attenuate cellular Ca^{2+} not only reduce the risk of excitotoxic damage but also form the basis for generation of oscillatory Ca^{2+} signals. The oscillations are ideal for encoding a large number of signals in terms of amplitude, duration, frequency and location of Ca^{2+} changes[1].

1.3 VGCC STRUCTURE, CLASSIFICATION AND FUNCTION

VGGCs are perhaps the best studied routes of extracellar Ca^{2+} entry and their presence defines excitable cells [20]. They were initially identified by Lux,

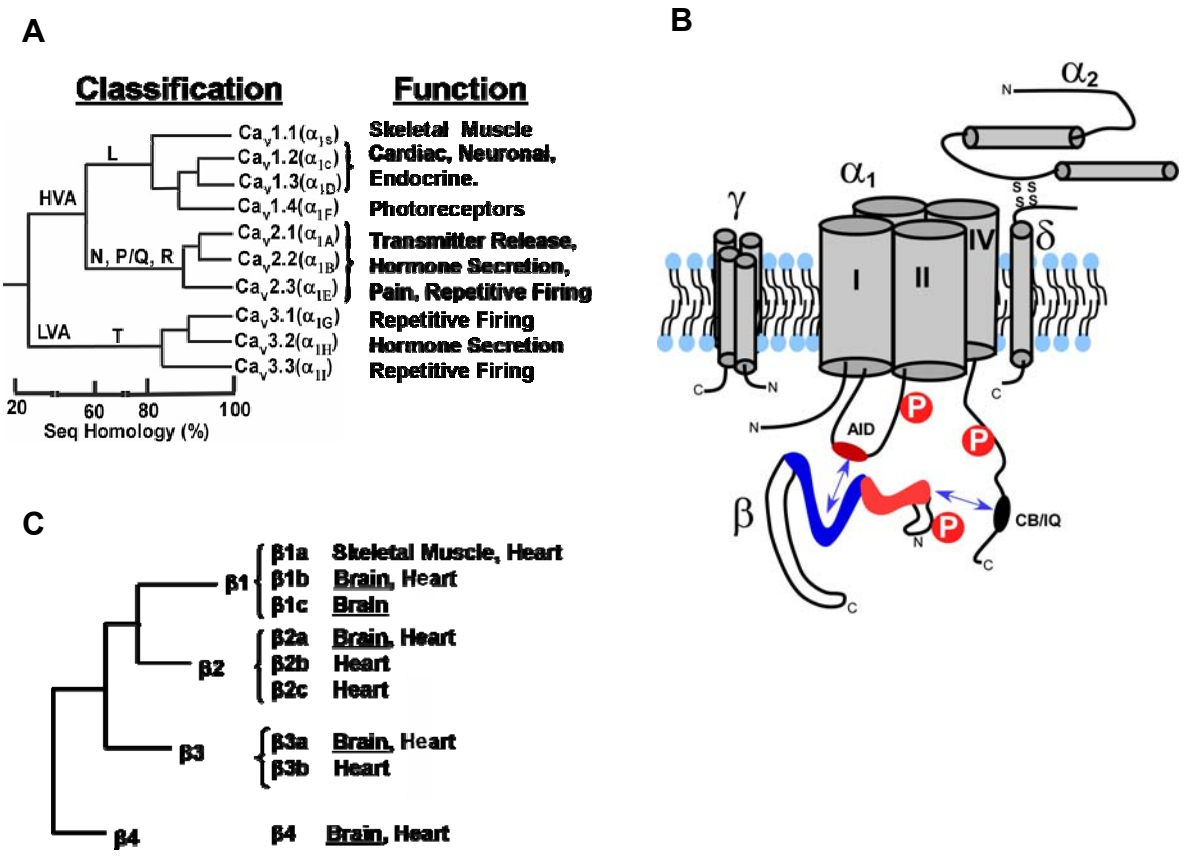

Figure 2. Generalized Structure and Diverse Functions of Voltage-Gated Calcium Channels.

(**A**). VGCCs are classified according to homology of α_1 subunits. The high-voltage activated (HVA) Ca^{2+} channels exhibit long-lasting (L-type) and intermediate-lasting (P/Q, N and R-type) currents. The low voltage-activated (LVA) Ca^{2+} channel family carries transient (T-type) currents. α_1 subunit isoforms are differentially expressed and perform multiple roles in various tissues.

(**B**). The α_1 subunit of HVA Ca^{2+} channels consists of four transmembrane domains (large cylinders), each containing 6 transmembrane helices and connecting loops, which have been omitted for simplicity. The δ subunit contains a single transmembrane domain and is linked by a disulfide bond to an extracellular α_2 subunit. The β subunit contains SH3 and GK domains and three variable regions. A hydrophobic pocket in the GK domain binds to the I-II linker of α_1; an SH3-containing region of β_{2a} also binds the CB/IQ domain of $Ca_v1.2\alpha_1$. Phosphorylations of α_1 and auxiliary subunits modulate VGCC function.

(C). Depicted here are the evolutionary relationships 9 of 18 differentially expressed **β** subunit gene products.

Carbone and Feldman as channels carrying two types of Ca^{2+} currents in mammalian sensory neurons. The authors classified the VGCCs into high-voltage activated (HVA) and low voltage-activated (LVA) Ca^{2+} channels according to their biophysical properties (Figure 2 A, also see [22]). Later, purification and molecular cloning of skeletal muscle HVA Ca^{2+} channels revealed that VGCCs are multimeric protein complexes consisting of a variety of α_1 subunits that form the Ca^{2+}-selective pore and auxiliary subunits (**Figure 2B and 2C**) [23, 24].The auxiliary subunits of VGCCs (β and α_2-δ) associate with and regulate trafficking, surface expression and activity of α_1 subunits.

VGCC Structure

Structure of the α_1 subunit. VGCCs contain an α_1 subunit that consists of four transmembrane domains (I through IV) with N and C-terminal tails (**Figure 2B**). Within each of the domains lie six α-helices (S1 through S6) that traverse the membrane and are linked by connecting loops. The four domains assemble to form a Ca^{2+} permeable pore. Membrane associated P-loops between S5 and S6 helices form the external pore lining, and contain determinants for Ca^{2+} selectivity. Helix 4 of each domain contains two positively charged amino acids thought to sense voltage for channel opening. The intracellular connecting loops and the long, alternately spliced N and C-terminal tails contain important regulatory sites. As will be discussed below, these include phosphorylation sites and binding sites for calmodulin, VGCC β subunits or other regulatory proteins. Even though the current understanding of VGCC structure is based on studies of

HVA Ca^{2+} channels structure, LVA Ca^{2+} channels share many of the features.

Structure of the β subunit: The α_1 subunits of VGCCs associate with cytosolic β subunits (**Figure 2B and 2C**). Four genes encode the β subunits, each with splice variants that differentially regulate channel activity and subcellular localization[25-27]. Crystal structures and homology mapping revealed that β subunits contain SH3 and guanylate kinase (GK) domains, similar to Membrane Associated Guanylate Kinase (MAGUK) family of scaffolding proteins[25, 27, 28]. The GK domain interacts with an 18-amino acid-long Alpha Interacting Domain (AID) in the I-II linker of α_1 subunits. β subunits lacking the GK domain can still associate with and regulate VGCC, implying that the β subunits can interact with multiple sites on the α_1. Indeed N-terminal domains on β_{2a} subunits can bind to the Ca^{2+}/CaM-binding CB/IQ domain at the C-terminus of α_1 in a manner dynamically regulated by Ca^{2+}/CaM[29]. The reported crystal structures of β subunits lack the N and C-terminal domains presumably because they are not well structured. Thus, the role of the N and C-terminal domains of the β subunit in structural assembly and regulation of the VGCCs remains less clear.

Structure of the α_2-δ and γ subunits: The α_2-δ is a combination of an ~180 kD extracellular, α_2 subunit linked with two disulfide bonds to a ~50 kD transmembrane δ subunit (**Figure 2B**). Both proteins originate from a single polypeptide that is subsequently cleaved into two proteins that remain associated. The γ subunit is made of 4 transmembrane helices. The precise sites

of interaction of α_1 and α_2-δ or γ subunits have not been determined.

VGCC Classification and Functions

VGCCs were originally classified into L, N, P/Q, R and T types based on their biophysical features and pharmacology but were recently reclassified based on homology of the α_1 subunits into Ca_v1, Ca_v2 and Ca_v3 subfamilies(**Figure 2A**) [22].

Ca_v1 (L-type) Ca^{2+} Channels

Voltage-activated L-type Ca^{2+} channels (LTCCs) generate Ca^{2+} signals for multiple physiological processes, including muscle contraction, neurotransmission, neuronal plasticity, dendritic arborization, gene expression and secretion[30]. The Ca_v1 subfamily of VGCCs form channels with long-lasting currents, and are sensitive to a class of clinically important, antihypertensive and antianginal drugs known as dihydropyridines. Dihydropyridine blockers such as nimodipine and (+)-Isradipine modify gating of LTCCs and also inhibit the channels in an activity-dependent manner. On the hand, other dihydropyridines such as BayK8644 activate LTCC by promoting an enhanced open state of the channel, known as mode 2 gating. LTCC are also selectively blocked by the phenylakylamine class of drugs, such as verapimil.

$Ca_v1.1$ LTCCs were the first VGCCs isolated from rabbit skeletal muscle. They form the predominant Ca^{2+} channels found in the Transverse (T-tubules) of the

skeletal myocytes and regulate excitation-contraction coupling. Point mutations in $Ca_v1.1$ are associated with hypokalemic periodic paralysis, malignant hyperthermia susceptibility and musclular dysgenesis in mice.

$Ca_v1.2$ LTCCs were initially cloned and isolated from rabbit heart and are now known to be expressed widely in the smooth muscle of blood vessels, intestines, lung and uterus. They are also found in many types of neurons and in endocrine cells of the pituitary and pancreas. $Ca_v1.2$ LTCCs exhibit a predominantly somatodendritic localization in neurons but are also found in spines [31-34]. Their well known functions include triggering of excitation-contraction coupling in heart and smooth muscle, excitation-transcription coupling in heart and neurons, action potential propagation in the sinoatrial (SA) and arterioventricular (AV) node, synaptic plasticity and hormone (e.g insulin) secretion[30]. $Ca_v1.2$ is also required for normal embryonic development and its knock out leads to death before embryonic day 14.5[35]. $Ca_v1.2$ is the main target of many cardiovascular dihydropyridines and may mediate the antidepressive effects of high doses of dihydropyridines[30]. Mutations of Cav1.2 are associated with Timothy syndrome, a disorder characterized by severe cardiac arrhythmias and mental disability, among other symptoms[17].

$Ca_v1.3$ LTCCs are HVA Ca^{2+} channels activated at relatively lower depolarizations than $Ca_v1.2$ [36]. In the brain, ~10% of LTCCs are composed of $Ca_v1.3$ [37], which are broadly present in medium spiny neurons, pituitary

neurons, pineal gland neurons, hippocampal neurons and other cell types[30]. Like their $Ca_v1.2$ counterparts, $Ca_v1.3$ LTCCs are primarily localized at proximal dendrites and cell bodies, even though they have also been detected in dendritic spines[30, 38]. $Ca_v1.3$ LTCCs are also expressed in sensory cells (photoreceptors, cochlear hair cells) and endocrine cells (pancreatic β cells, adrenal chromaffin cells)[30]. Outside the neuroendocrine system, they are expressed at low density in smooth muscle and the heart, primarily in the atrial muscle, the SA node and atrioventricular AV node. Functionally, $Ca_v1.3$ channels mediate neurotransmitter release in sensory cells, control cardiac rhythm, mood, behavior and hormone secretion[30]. The importance of $Ca_v1.3$ LTCC functions is underscored by their contribution to parkinsonism[39, 40] and the association of their mutations with congenital deafness, as well as SA and AV node dysfunction[41].

$Ca_v1.4$s are relatively less studied and are found in the retina (in photoreceptors and bipolar cells) where they control neurotransmitter release. They are also present in the spinal cord and in cells that are not traditionally considered excitable such as lymphoid cells of the immune system [30].

Ca_v2 (N, P/Q, and R-type) Ca^{2+} Channels

N, P/Q and R-type Ca^{2+} currents were initially identified as a non-L-type neuronal component of high voltage activated Ca^{2+} currents that was not blocked by

dihydropyridines [22, 30]. $Ca_v2.1$ (P/Q-type) channels exhibit two types of currents. The P-type current was originally identified as the dihydropyridine insensitive, slowly inactivating component of HVA Ca^{2+} channel current in cerebellar **P**urkinje cells. A faster inactivating current was also identified in cerebellar granule cells and named **Q**-type channels. P/Q channels are broadly distributed in neurons (presynaptic terminals, dendrites and cell bodies), heart, pancreas and pituitary. They are most known for stimulation of neurotransmitter release in the central nervous system (CNS) and at neuromuscular junctions, and triggering excitation-secretion coupling in pancreatic β cells. $Ca_v2.2$ (N-type) channels are highly expressed in the presynaptic termini, dendrites and cell bodies of neurons but are also present in other tissues such as adrenal glands. They function in neurotransmitter release in central and sympathetic synapses, in sympathetic regulation of circulatory system[22, 30]. $Ca_v2.3$ R-type channels carry the residual neuronal currents that are resistant to block of L, P/Q and N-type channels. They are widely distributed in neurons (cell bodies, dendrites and some presynaptic terminals), heart, testes and the pituitary glands. They control neurotransmitter release, repetitive neuronal firing, post-tetanic potentiation and neurosecretion[22, 30].

Ca_v 3 (T-type) Ca^{2+} Channels

Ca_v3 (T-type) VGCCs were identified as channels underlying a transient current that activated at low voltages (~-50mV). Molecular cloning of Ca_v3 revealed three conserved, alternatively spliced, genes of Ca_v3 channels $Ca_v3.1$, 3.2 and 3.3 with

largely overlapping expression in the peripheral tissues including heart, kidney, smooth muscle, sperm and many endocrine organs. Unlike the channels in the HVA Ca^{2+} channel family, there is little evidence that Ca_v3s (LVA Ca^{2+} channel family) are stably associated with auxiliary subunits[22, 30, 42]. The ability of Ca_v3 channels to open transiently at low potentials may enable them to contribute to Ca^{2+} entry at or near resting membrane potentials. The resulting low-threshold Ca^{2+} spikes can in turn trigger Na^+ action potentials important in synchronized activity in the thalamus. Indeed Ca_v3s are thought to be essential for neuronal burst firing, which in turn correlates with pathological Ca^{2+} waves generated in seizures. $Ca_v3.2$ mutations have been identified in certain human absence seizures and have also been implicated in generation of spontaneous sinoatrial node rhythm. Thus Ca_v3 channels are drug targets for epilepsy, chronic pain and sleep disorders [22, 30, 42].

The expression and function of β subunits

Even though the α_1 subunits are the major determinants of VGCC classification (**Figure 2B**) and function, the identity of their auxilliary subunits can markedly alter their properties. Each of the β subunit genes generates protein variants that are differentially expressed and regulate channel activity and subcellular localization[25-27]. In the heart, β_2 is the predominant isoform but β_1, β_3 and β_4 are also present[26, 43]. In the adult brain β_3 and β_4 show stronger overall staining than β_1 and β_2, and together comprise about ~70% of total the β subunits in adult forebrain [25, 44-46]. In addition to their differential tissue

localization, the β subunits also exhibit differences in subcellular localization. Variants of β_1 and β_2 localize to the membrane via N-terminal acidic residues and palmitoylation sites respectively. β_3 and β_4 are largely cytosolic but β_4 also localizes to the nucleus to regulate gene expression[47, 48].

The differential expression and subcellular localization of α_1 subunits and β subunits likely yields a set of VGCCs diverse in their composition. Within the VGCC complex, α_1-β pairing is likely to be controlled by relative subunit concentrations, affinities, number of binding sites on α_1, and subcellular localization of β (and α_1 subunits)[25]. While there is no evidence of exclusive pairing of α_1 and β subunit isoforms, a majority of N-type and P/Q-type channels have been reported to be in β_3 and β_4 immunoprecipitates[25]. Regardless of their identity, the β subunits enhance functional expression of VGCCs, an effect attributed to their regulation of VGCC surface expression, open probability or voltage-dependence of activation. All β subunit isoforms shift the activation of VGCCs towards negative potentials but some biophysical properties of the channels are differentially modulated by β subunit isoforms (**see Regulation of VGCCs in the following section**).

1.4 REGULATION OF VGCCS

There are multiple levels of VGCC regulation. The first level of control of VGCC activity is through variation of the intrinsic composition of the channels, which arguably is the limiting determinant of the maximal and minimal Ca^{2+} that enter

through channel. The combination of diverse α_1 and auxiliary subunit variants allows for immense complexity and variability of intrinsic VGCC function. A second level of VGCC control involves extrinsic factors that combine with the intrinsic regulatory mechanisms to increase or reduce Ca^{2+} entry via VGCCs. The ability of α_1 and β subunits to recruit additional regulatory proteins is particularly important for diversifying the set of extrinsic mechanisms that impinge on the channel.

Regulation of VGCCs by Voltage

Voltage-dependent Inactivation and Facilitation: Membrane depolarization induces a progressive transition of activated VGCCs to non-conducting states[49-51]. This voltage-dependent inactivation (VDI) response to stimulation may be important in restricting Ca^{2+} entry to prevent cellular Ca^{2+} overload. Impairment of VDI can lead to the deleterious consequences of abherrant Ca^{2+} entry. For example, a $Ca_v1.2\alpha_1$ mutation that leads to reduced VDI, and thereby enhanced Ca^{2+} entry into cells results in arrhythmia, deficits in learning and other problems in Timothy Syndrome[17, 52]. In contrast to VDI, VGCC currents are enhanced when cells are stimulated with a train of repetitive depolarizations or single strong depolarizations[53, 54]. Typically, these changes in voltage drive VGCCs from normal gating patterns to longer or more frequent openings, a process known as voltage-dependent facilitation (VDF). VDI and VDF are thought to occur to all VGCCs even though they have been most extensively studied in $Ca_v1.2$, 2.1 and $Ca_v2.2$ channels.

Mechanism of VDI and VDF: The precise molecular mechanisms for both VDI and VDF are unclear [23]. VDI requires the α_1 C-terminus, IS6 (the helix 6 of domain I, which lines the pore of the channel (**see section on VGCC structure**) and the AID but the N-terminus and other domains have also been implicated[55]. The C-terminus of $Ca_v2.1$ contains a CaM binding domain that increases both the extent and rate of VDI [23]. In $Ca_v2.2$ channels VDI has been proposed to involve disinhibition of G-protein block [23]. The β subunits differentially decrease VDI: the β_{2a}, β_{2e} and β_4 variants reduce VDI more markedly than β_1 and β_3. The molecular basis for the differential modulation of VDI by β subunits is not clear. However, localization of the β subunits to the α_1 at the surface appears to be an important factor as mutation of the palmitoylation sites on β_{2a} severely impairs its effect on VDI. Furthermore, β subunit interaction with the IS6-AID domain of the α_1 subunit are also important in VDI. Like VDI, VDF is also linked to conformational changes involving the IS6, AID domains and the β subunit, but to my knowledge the β isoform dependence of VDF has not been studied[56]. Finally, phosphorylation is another mechanism of modulating voltage-dependent LTCC regulation but its role is described in a later section.

Regulation of VGCCs by Ca^{2+}

CDI and CDF: Ca^{2+} ions regulate their own entry into cells by complex and poorly understood mechanisms[57, 58]. Increases in intracellular Ca^{2+} during depolarization enhance VGCC transition to less conducting states; this phenomenon is known as Ca^{2+}-dependent inactivation (CDI). The relative

importance of CDI and the VDI discussed in the previous section remains unclear, but CDI is thought to be faster and therefore more important in determining VGCC inactivation during depolarization[23]. CDI has been shown in the major VGCCs, including $Ca_v1.2$, 2.1, 2.2 and 2.3 channels[57, 58]. CDI of LTCCs is thought to be induced by Ca^{2+} entry through the channel from the extracellular space and Ca^{2+} release from the SR. The two sources of Ca^{2+} appear to differentially affect CDI: extracellular Ca^{2+} entry is linked to a slower component of CDI while Ca^{2+} from the ER is linked to a faster component[23]. These observations suggest that the rate of CDI onset may depend on the amplitude of Ca^{2+} increases as SR release contributes more Ca^{2+} than LTCC-mediated influx. Small increases in basal Ca^{2+} or transient, repeated depolarizations activate Ca^{2+}-dependent facilitation (CDF) processes. CDF has been observed in $Ca_v1.2$, $Ca_v1.3$ and $Ca_v2.1$ and may play roles in augmenting Ca^{2+} for excitation-contraction coupling, excitation-transcription coupling and for controlling neurotransmitter release in central nervous system synapses[23, 47, 48].

Molecular Mechanisms of CDI and CDF: CDI of $Ca_v1.2$ is less sensitive to Ca^{2+} buffering than that of $Ca_v2.1$, $Ca_v2.2$, $Ca_v2.3$, meanwhile the CDF of $Ca_v2.1$ is relatively insensitive to Ca^{2+} buffering. These observations suggest the existence of distinguishable molecular determinants in the Ca^{2+} channel subunits. Most work on the molecular determinants of CDI and CDF has been done on $Ca_v1.2$ and $Ca_v2.1$. The molecular determinants of CDI and CDF include sequences of

the channel α_1 subunits, the nature of associated β subunits, direct Ca^{2+} binding and CaM binding to the α_1 subunit [55, 57, 58].

CaM is the most extensively studied regulator of CDI and CDF. It is the primary Ca^{2+} sensor for CDI in $Ca_v1.2$, 2.1, 2.2 and 2.3 and for CDF in $Ca_v1.2$ and 2.1[23]. Binding of Ca^{2+}/CaM to the C-terminus of $Ca_v1.2\alpha_1$ modulates LTCCs, likely through a two-step process involving an initial binding of Ca^{2+}-free ApoCaM (priming) to the channel and a subsequent binding Ca^{2+} to ApoCaM. The C-terminus of $Ca_v1.2$ contains a consensus ApoCaM (and Ca^{2+}/CaM) binding IQ motif (IQXXXRGXXXR, where the first letter is usually an I, L or V) that is critical for both CDI and CDF[58]. A Ca^{2+} binding (CB) domain N-terminal to the IQ domain also binds Ca^{2+}/CaM, but with a lower affinity relative to the IQ domain. $Ca_v2.1$ also contains an IQ-like motif and a CBD domain that has been shown to mediate both CDI and CDF. Thus, CaM generally regulates both CDI and CDF.

Coupling of CaM to the Regulatory Machinery: Even though the C-terminal role of CaM in CDI and CDF is well demonstrated, it is not clear how CaM binding is coupled to the inactivation or facilitation machinery of VGCCs[58]. It has been suggested that, like VDI, CDI and CDF involve interaction of the C-terminus, the IS6 helix and the AID and the β subunits[55, 57, 58]. However, other parts of the channel may still play important roles in Ca^{2+}-dependent regulation. Dick and colleagues recently identified a Ca^{2+}/CaM binding sequence at the N-terminus of VGCCs that determines whether CDI responds to local or global increases in

Ca^{2+} [59]. Thus, CDI and CDF involve multiple parts of the channel; the various parts may exist close to one another in the functional LTCC complex and in concert induce CDI or CDF.

Alternative mechanisms of Ca^{2+}-dependent VGCC regulation: The absence of CDI in $Ca_v1.4$ despite the presence of CaM binding sites raised the possibility that CaM binding might not be sufficient for CDI[58] and alternative mechanisms may mediate Ca^{2+} regulation of VGCC. One possibility is that dynamically regulated movements of the channels or surrounding macromolecular complexes play important roles in modulating the channels. For example, a Ca^{2+}-dependent disruption of the cytoskeleton has been proposed to mediate CDI partly because cytoskeletal stabilizers Taxol and Phalloidin reduce CDI[57].

I hope it is clear from the preceeding discussion that α_1 subunit-based mechanisms have been the primary focus of studies of CDI and CDF. However, VGCC β subunits also critically modulate VDI, CDI and CDF through mechanisms that are currently unclear [47, 55, 56, 60]. The effects of the β subunit on CDF may be linked to a reduction of VDI that then unmasks CDF by Ca^{2+}/CaM[23]. Additionally the β subunit may also reveal channel facilitation by antagonizing the inhibitory CaM binding to the CB/IQ[29]. Structurally, the role of β subunit in CDI or CDF has been linked to its interaction with a rigid IS6-AID[55]. The requirement of the β subunit for normal CDI and CDF, and its interactions with both termini of the α_1 and to the IS6-AID emphasizes its importance in

structural assembly of the channel for normal regulation. Finally, phosphorylation of the α_1 and β subunit is also important in Ca^{2+}-dependent regulation of VGCCs.

Regulation of VGCCs by Phosphorylation and Dephosphorylation

Phosphorylation and dephosphorylation of proteins is an important means of modifying their structure, function, localization and other properties. The antagonistic regulation of VGCCs by protein kinases and phosphatases appears to be a general phenomenon in animals (reviewed in [23, 57] and [24]).

Regulation of VGCCs by Phosphorylation

VGCCs are phosphorylated by at least 8 kinases. Regulatory phosphorylations of all VGCCs by various kinases are summarized in **Table 1**. LTCC phosphorylations by PKA, PKC and CaMKII have been most extensively investigated and are therefore discussed in detail in this chapter.

LTCC regulation by PKA: In initial studies of VGCC regulation by phosphorylation, PKA pathway activation in neuronal cell-types markedly decreased CDI (see review in reference[57]). Subsequent studies showed PKA potentiates $Ca_v1.1$ activity and phosphorylates both the α_1 and β subunits *in vitro* and in myotubes (fused skeletal myocytes)[24]. However, the PKA phosphorylation sites on $Ca_v1.1$ α_1 (Ser687, 1757 and 1854) and of β_1 (Ser182 and Thr205) have not been linked to any functional modulation of the channels.

Subunit	Phosphorylation Site	Kinase	Effect on Calcium channel Activity	References/Comments
$Ca_v1.1\alpha_1$	S687 S1857 S1854	PKA	Not determined	24
$Ca_v1.2\alpha_1$	S439	CaMKII	Increased	61 Site created by TS mutation
	S528	PKG	Not determined	62
	S533	PKG	Decreased	62
	S1517	CaMKII	Increased	61, 63
	S1575	CaMKII	Increased	63
	S1829	LIF	Increased	64
	S1928	PKA	Increased/No effect	65-68 Controversial
		PKC	Not determined	69
		PKG	No effect	62
	Y2122	c-Src	Increased	71
	T1604	CaMKII	Slowed rundown	70
$Ca_v1.3\alpha_1$	S81	PKC	Decreased	72
	S1486	CaMKII	Increased	38
	S1743	PKA	Increased	73 β_3 dependent in 74
	S1816	PKA	Increased	73
	S1964	PKA	Increased	74 β_{2a} dependent
$Ca_v2.2\alpha_1$	S422	PKC CaMKII	Increased	24
$Ca_v3.2\alpha_1$	S1198	CaMKII	Not determined	75,76 Dissociates CaMKII
$Ca_v\beta_{1a}$	S182 T205	PKA	Not determined	24
$Ca_v\beta_{2a}$	S459	PKA	No effect	77,78
	S496	PKG	Decreased	62
	S478	PKA	Increased	77,78
	S479	PKA	Increased	77,78
	T498	CaMKII	Increased	79
	S574	AKT	Increased $Ca_v1.2\alpha_1$ Trafficking	80
	S625	AKT	Reduced $Ca_v1.2\alpha_1$ Degradation	81

Table 1. VGCC Regulation by Phosphorylation.

A variety of protein kinases regulate VGCCs by phosphorylation of the α1 and β subunits ([24] and [38, 61-81]). Specific phosphorylation sites have been shown to increase or decrease VGCC activity. The protein phosphatases responsible for dephosphorylating a limited number of sites have been identified. In some cases, localization of enzymes to VGCCs is important in regulation of the channels, possibly by modulation of phosphorylation.

PKA-dependent phosphorylation of $Ca_v1.2$ has been more extensively investigated than that of $Ca_v1.1$. PKA phosphorylates Ser1928 of $Ca_v1.2$. The role of this phosphorylation on LTCC regulation remains unclear. Phosphorylation of Ser478/9 of the β_{2a} subunit by PKA also enhances $Ca_v1.2$ currents (reviewed in [24]). PKA enhances $Ca_v1.3$ LTCC activity through a mechanism involving Ser1743 and Ser1964, though it is still not known if PKA directly phosphorylates these residues[74]. Interestingly, the identity of the β subunit associated with $Ca_v1.3$ determines whether PKA utilizes Ser1743 or Ser1964 in regulation of $Ca_v1.3$[74].

LTCC regulation by PKC: The activation of PKC by Gq coupled receptors or by direct action of phorbol esters, has produced contradictory effects (decreases or increases) in LTCC activity. PKC phosphorylates the α_1 subunit of $Ca_v1.2$ and at least one β subunit. The PKC-dependent phosphorylation of $Ca_v1.2\alpha_1$ subunit at Ser27 and Ser31 decreases channel activity. The $Ca_v1.2\alpha_1$ Ser1928 phosphorylated by PKA is also a PKC substrate. $Ca_v1.3$ α_1 phosphorylation by PKC at Ser 81 also reduces channel activity. For more detailed review of VGCC regulation by PKC, I recommend reading [24].

LTCC regulation by CaMKII: regulation of LTCCs by CaMKII is the main subject of this thesis and thus is omitted from this section and covered extensively later (**see section 1.6**)

Regulation of VGCCs by dephosphorylation

Much like role of phosphorylation, the role of dephosphorylation in VGCC regulation has been most intensely investigated in LTCCs, particularly in the $Ca_v1.2$ subtype. Purified protein phosphatases were initially noted to reverse LTCC activation by β adrenergic receptors, without affecting basal channel activity (reviewed in 23 and 24). This implied the absence of significant basal phosphorylation of LTCCs, but several lines of evidence indicate that protein phosphatases are active at basal conditions. For example, enhancement of dephosphorylation at basal conditions increases in the rate of VGCC inactivation in mollusks and phosphatase inhibition reduces rundown of LTCCs. The above studies and other details on phosphatase regulation of LTCCs are reviewed in [23, 24, 57].

A note on other mechanisms of LTCC regulation: While phosphorylation and dephosphorylation are important regulatory LTCC processes, LTCCs are also regulated by a variety of other proteins and pathways not detailed here (reviewed in [24]. These include Ca^{2+} binding proteins, which modulate neuronal $Ca_v1.2$ independently of Ca^{2+}/CaM[48]. Binding of the Rem and Gem G-proteins also modulates LTCC function[82]. Lastly, proteolytic cleavage of the C-terminus of $Ca_v1.2$ also enhances the activity of the channels, likely by disrupting an autoinhibitory mechanism involving the C-terminus of the α_1 subunit[83].

1.5 THE IMPORTANCE OF LOCALIZED SIGNALING COMPLEXES IN LTCC REGULATION

It has been widely postulated that cells concentrate and juxtapose related signaling molecules to promote efficient, specific and finely-tuned signaling (reviewed in [84]). The prototypical β-AR regulation of LTCCs has been shown to involve formation of a signalosome containing the receptor, an adenylyl cyclase, PKA and at least one A-kinase Anchoring Protein (AKAP) [23, 24, 68, 85, 86]. In neurons, MAP2B targets PKA to modulate LTCCs. Studies of the PKA targeting mechanisms have popularized the view of cellular signaling as a combination of descrete and localized events and this view is now broadly applied to signaling by multiple enzymes. For example, PKC can be directly anchored at the α_1 subunit of $Ca_v1.2$ but can also be targeted there by an AKAP150[24]. Many LTCC- complexes also contain phosphases to counteract the effects of the associated kinases. LTCCs associate and copurify with protein phosphatases, and PP2A associates with $Ca_v1.2\alpha_1$ directly or via AKAP150, along with PKA and β-AR[86]. The same AKAP also targets PP2B to neuronal LTCCs to reverse localized PKA-mediated upregulation of LTCC function[86]. Therefore, both kinases and phosphates are targeted to locally control the phosphorylation of VGCCs, and to fine tune Ca^{2+} signals at the point of entry. The paradigm of PKA targeting to LTCCs and β-adrenergic receptors to promote fast, efficient and specific modulation of Ca^{2+} signaling has provided a reasonable framework for understanding CaMKII-dependent regulation of LTCCs that is the subject of the rest of this thesis.

1.6 CAMKII: STRUCTURE, REGULATION, FUNCTION AND DYNAMIC LOCALIZATION

A comprehensive discussion of this subject is beyond the scope of this document but those interested are referred to detailed reviews[87, 88]. The discussion hereafter is focused on aspects of CaMKII most relevant to this thesis.

CaMKII Structure and Regulation

CaMKII is a ubiquitously distributed serine-threonine kinase with multiple functions[88]. Four alternately spliced gene products of CaMKII have been identified in vertebrates (α, β, δ and γ). The four CaMKII isoforms share more than 80% amino acid identity, and have similar structure and function. Expression of the resulting CaMKII isoforms varies between tissues and during development [88, 89]. CaMKIIδ is the main isoform expressed in heart whereas the α and β predominate in the brain and together constitute ~1% of total protein in forebrain and as much as 2% of protein in the hippocampus. Within the CNS, CaMKIIα is expressed in some neuronal subtypes but CaMKIIβ is found in most neurons and in glia. The δ and γ isoforms of CaMKII are also present in select regions of the brain but their roles are undefined.

Individual CaMKII subunits each contain an N-terminal catalytic domain, an adjacent autoinhibitory domain (AID), and a calmodulin binding domain[88, 90]. The subunits also contain C-terminal association domains through which the subunits form two hexameric rings that stack to form dodecameric holoenzyme

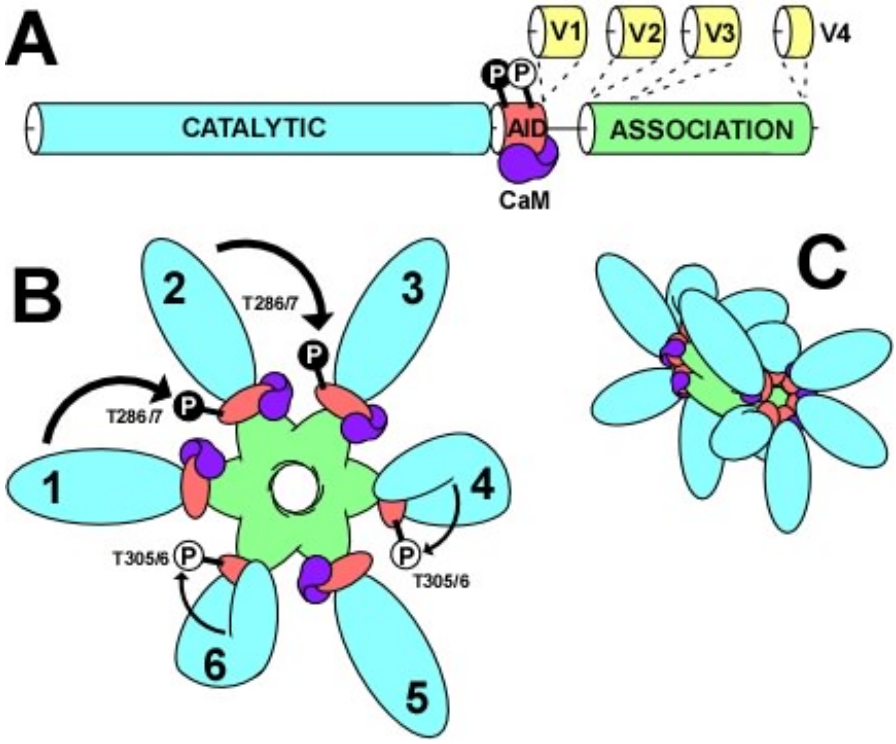

Figure 3. Structure and Regulation of CaMKII.
CaMKII subunits (**A**) consist of catalytic, autoregulatory, alternately spliced variable (V1-4) and association domains. CaMKII subunits oligomerize into hexameric rings (**B**) which stack pair-wise to form a dodecameric holoenzyme (**C**). The catalytic domain, conserved in Ser-Thr kinases, contains nucleotide and substrate-binding lobes. The latter is partly occupied by the region surrounding $Thr^{286/7}$ (the autoinhibitory domain, AID) at basal Ca^{2+} levels (**B**). Ca^{2+}/Calmodulin (Ca^{2+}/CaM) activates each subunit by displacing the AID. Binding of Ca^{2+}/CaM to adjacent subunits results in int**er**-subunit autophophorylation at $Thr^{286/7}$, which enhances CaMKII affinity for Ca^{2+}/CaM and confers Ca^{2+}-independent activity. Int**ra**-subunit autophosphorylation at $Thr^{305/6}$, following dissociation of Ca^{2+}/CaM, prevents binding of Ca^{2+}/CaM and other proteins. *Adapted from [91]*

(Figure 3). Under some circumstances holoenzymes of CaMKII can assemble via dimerization of catalytic and autoregulatory domains into supramolecular structures[88, 90].

At basal levels of Ca^{2+}, the AID of CaMKII subunit binds the catalytic site and inhibits it. The autoinhibition is relieved by displacement of the AID upon Ca^{2+}/CaM binding, leading to activation of the kinase. Coincident binding of Ca^{2+}/CaM to adjacent subunits of a CaMKII holoenzyme leads to intersubunit autophosphorylation at Thr^{286} (Thr^{287} in β, δ and γ), which increases their affinity for Ca^{2+}/CaM by more than one thousand-fold and also renders them Ca^{2+}-independent. Intra-subunit autophoshorylation at $Thr^{305/6}$ occurs at basal Ca^{2+} levels or following dissociation of Ca^{2+}/CaM, and prevents association/activation of the kinase until the two sites are dephosphorylated. The level of Thr286 autophosphorylaton increases with frequency and duration of Ca^{2+} oscillations *in vitro*[92]. In neurons, increasing frequency of action potentials enhances CaMKII autophosphorylation [93] and Ca^{2+} spikes generated by different patterns of neuronal stimulation induce redistribution of CaMKII[94, 95]. Similarly, increasing heart rate or stimulation of cardiomyocytes with hormones stimulates CaMKII autophosphorylation and redistribution [96, 97]. These studies raise the possibility that CaMKII integrates and encodes Ca^{2+} dynamics at and around Ca^{2+} channels into molecular states of activation, autophosphorylation and localization. These features of CaMKII are ideal for interpreting and storing a

molecular memory of prevailing Ca^{2+} dynamics associated with neuronal activity and for regulating cardiac contractility.

CaMKII Function

CaMKII Substrates: The earliest known substrates of CaMKII include glycogen synthase, synapsin, myosin light chain, casein, MAP-2 and tryptophan hydroxylase [88, 98]. Over the years a wide range of CaMKII substrates have been characterized, many of which contain the mimimal consensus sequence Arg-X-X-Ser/Thr [98] or the more restrictive Hyd-X-Arg-NB-Ser/Thr-Hyd [99]. Substrates of CaMKII in the heart include ryanodine receptors, IP3 receptors and other proteins involved in gene expression and Ca^{2+} cycling. They are discussed in a previous thesis[100] and review[101] and will not be covered here. The focus here is on CaMKII substrates in neurons. CaMKII phosphorylates proteins in presynaptic termini (such as $Ca_v2.1$, 2.2, 2.3 and synapsin) and at least 30 proteins in the postsynaptic spine are known or putative CaMKII substrates [102, 103]. The postsynaptic proteins include α-amino-3 hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptors (AMPARs), N-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARs), LTCCs and RTCCs, which control basic neurotransmission or plasticity. The availability of a wide range of CaMKII substrates involved in Ca^{2+} signaling may enable the enzyme to control acute and long-term function of cardiomyocytes, neurons and other cell types by phosphorylation.

Role of CaMKII in Normal Cardiac Function

Cardiac excitation-contraction coupling is regulated by actions of CaMKII on LTCCs, RYRs and the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) [104]. CaMKII may also influence LTCC-dependent initiation of action potentials at the sinoatrial node[105] or their conduction at the atrioventricular node[106, 107]. Thus, CaMKII can acutely modulate cardiac excitability and contractility, and also plays roles in long-term Ca^{2+} homeostasis and gene expression[104, 107].

Role of CaMKII in heart disease

Sustained increases in CaMKII expression or activity contribute to long-term alterations in Ca^{2+} homeostasis and abnormal remodeling of the heart (reviewed in [101, 107, 108]). Briefly, CaMKII expression or activity is increased in patients and animal models of structural heart disease and arrhythmias. Our lab has shown that CaMKII inhibition attenuates arrhythmias and structural damage in mouse models of heart disease[109, 110]. Thus, CaMKII inhibition is a viable strategy for treatment of certain forms of heart disease.

Role of CaMKII in Normal Neuronal Function and in Disease

Role in Normal Functions: CaMKII regulates several neuronal processes that underlie synaptic transmission, plasticity and spatial learning. It is required for activity-dependent modification of synaptic transmission (LTP and LTD), cellular models for learning and memory [111-114]. CaMKII also regulates neurotransmitter synthesis and release[115], dendritic morphology[116] and

neurite outgrowth[117, 118]. *In vitro* and cellular studies suggest actions of CaMKII are related to its ability to sense and transduce dynamic changes in cytosolic Ca^{2+} into biochemical alterations of key synaptic proteins [92, 93, 96, 119-122]. For example, CaMKII phosphorylates AMPARs to enhance their function in LTP [119, 121] and also regulates Ca^{2+} influx via the NMDAR[120].

Role in Disease: In the brain, hyperactivation of LTCCs by CaMKII is implicated in Timothy Syndrome, the multi-organ human genetic disorder whose symptoms include mental disability[17, 52]. Furthermore, LTCC-dependent spine loss in striatal medium spiny neurons[40] occurs in parallel with CaMKII overactivation in animal models of parkinsonism[123, 124]. Neuropathological conditions such as ischemia and excitotoxicity promote CaMKII accumulation at postsynaptic densities (PSDs or protein-rich specializations found in over 80% of dendritic spines) [125-128].

In mouse models of Angelman's syndrome, a human disorder characterized by mental disability, ataxia and seizures, levels of CaMKII at the PSD are reduced[129]. This decrease correlates with increased $Thr^{305/306}$ autophosphorylation, which is known to reduce synaptic CaMKII association[130]. Mutation of $Thr^{305/6}$ to Asp mimics phosphorylation at these sites, resulting in reduced PSD-associated CaMKII and deficits in spatial learning[130]. Conversely, a mutation of $Thr^{305/6}$ to Ala rescues neurological deficits in Angelman's syndrome [131]. Similar behavioral defects were observed

in mice in which local dendritic synthesis of CaMKII was blocked[132]. Thus, misregulation of CaMKII expression or localization is associated with neurological disease.

1.7 Mechanisms of Subcellular Targeting of CaMKII

The ability of CaMKII to integrate past neuronal experience with prevailing stimuli into activation, autophosphorylation and localization states appears to be important for adaptive and maladaptive changes that affect neuronal response to future stimuli. CaMKII migrates to substrates and sites of Ca^{2+} entry, where its actions on receptors and channels may induce changes in synaptic function and morphology, and ultimately in physiology.

CaMKII Targeting to postsynaptic sites: Neuronal activity, *in vitro* or *in vivo*, stimulates rapid Ca^{2+}-dependent, translocation of CaMKII to postsynaptic sites and to the PSD [94, 95, 133, 134]. Ca^{2+} entry following NMDA receptor activation and the consequent binding of Ca^{2+}/CaM or autophophorylation of CaMKII is a key mechanism of driving CaMKII to the synapse. Stable association of CaMKII at the synapse depends on intensity of depolarization and level of glutamate [95], implying that CaMKII can sense and respond dynamically to varying Ca^{2+} signals. The levels of CaMKII at the PSD may also depend on its dynamic interactions with various **CaMK**II **A**ssociated **P**roteins (**CaMKAP**s) at the PSD, which in some cases are regulated by Ca^{2+}/CaM or autophosphorylation(reviewed in[135, 136]).

Examples of CaMKAPs include Densin-180, α-actinin and subunits of the NMDA receptor (NR1, NR2A and NR2B).

CaMKII Targeting to the NMDAR: The mechanism and regulation of CaMKII binding to NR2B subunits has been investigated in a few studies. Displacement of the CaMKII autoinhibitory domain after Ca^{2+}/CaM binding to CaMKII or autophosphorylation at Thr^{286} exposes the catalytic site and adjacent regions for interaction with the cytosolic C-terminal tail of NR2B. The major CaMKII binding domain of NR2B (residues 1290-1309) is conserved with the CaMKII autoinhibitory domain; phosphorylation of Ser^{1303} substantially diminishes NR2B binding to CaMKII *in vitro*, similar to the block of AID binding by $Thr^{286/7}$ autophosphorylation. These findings suggest interaction of CaMKII with NR2B-containing NMDARs is dynamically regulated by Ca^{2+}/CaM binding and autophosphorylation following synaptic activity. Indeed, Thr^{286}-autophosphorylation is required for CaMKII-dependent regulation of NR2B-containing NMDARs[120]. Disruption of CaMKII association with the NR2B subunit of NMDARs interferes with LTP in cultured neurons[137] and overexpression of the NR2B C-terminus containing a CaMKII binding region impairs learning in whole animals[138]. Thus NMDAR signaling appears to be critically modulated by both downstream and feedback actions of CaMKII near the channel.

1.8 DYNAMIC CAMKII TARGETING TO REGULATE LTCCS

LTCCs are the primary means of Ca^{2+} entry into cardiomyocytes and control both acute cardiac functions such as contraction but also long-term remodeling and gene expression. They also coexist with NMDARs in neuronal dendritic spines where they function in parallel with NMDARs to control Ca^{2+} entry, neuronal plasticity and gene expression. Like NMDARs, downstream LTCC signaling is modulated by CaMKII. Furthermore, $Ca_v1.2$ channels have been recognized as potential CaMKII regulatory targets since their identification as CaMKII substrates nearly 22 years ago[139]. CaMKII inhibition impairs voltage-dependent facilitation of $Ca_v1.2$ in heterologous cells [63, 140] and Ca^{2+}-dependent facilitation in both heterologous systems and cardiomyocytes(**Figure 4**)[141, 142]. CaMKII also potentiates $Ca_v1.2$ following Gq-coupled receptor activation in cardiomyocytes[97] and $Ca_v1.3$ following insulin growth factor receptor activation in a neuronal cell line[38]. Therefore, under normal conditions, LTCC facilitation may augment cellular Ca^{2+} signals necessary for adaptive physiological responses such as fight or flight.

Excessive LTCC activity, Ca^{2+} entry and CaMKII activation are associated with cardiac arrhythmias and other forms of heart disease[17, 52, 107]. LTCCs are themselves important therapeutic targets[143]. The Timothy Syndrome mentioned earlier is associated with a novel mutation of the $Ca_v1.2\alpha_1$ subunit that facilitates LTCC activity, because the G406R mutation creates a CaMKII consensus phosphorylation site at S409 in $Ca_v1.2$ α_1 [61].

Like NMDARs, LTCC modulation by Ca^{2+}/CaMKII-dependent feedback mechanisms appears to involve localized actions of CaMKII. Drawing on relevant parallels from CaMKII-dependent NMDAR regulation, this thesis examines the mechanism of CaMKII targeting to modulate LTCCs in neurons and cardiomyocytes.

Importance of CaMKII-targeting in LTCC regulation

LTCC facilitation by membrane depolarization or Ca^{2+} has been proposed to require CaMKII actions near the channel[140, 141, 144]. In support of this notion, the open probability of LTCCs in membrane patches—typically smaller than 1 μm in diameter[145]— that are excised from rabbit cardiomyocytes is enhanced by activation of endogenous CaMKII within the patches[144]. Cytoskeletal disruption prior to excision of the patches specifically inhibits CaMKII-dependent, but not PKA-dependent regulation of cardiac LTCCs[144]. These findings suggested that CaMKII actions on LTCCs are dependent on scaffolding in the vicinity of the channels and are consistent with the localized nature of CaMKII-dependent LTCC facilitation revealed by chelation of Ca^{2+} microdomains with

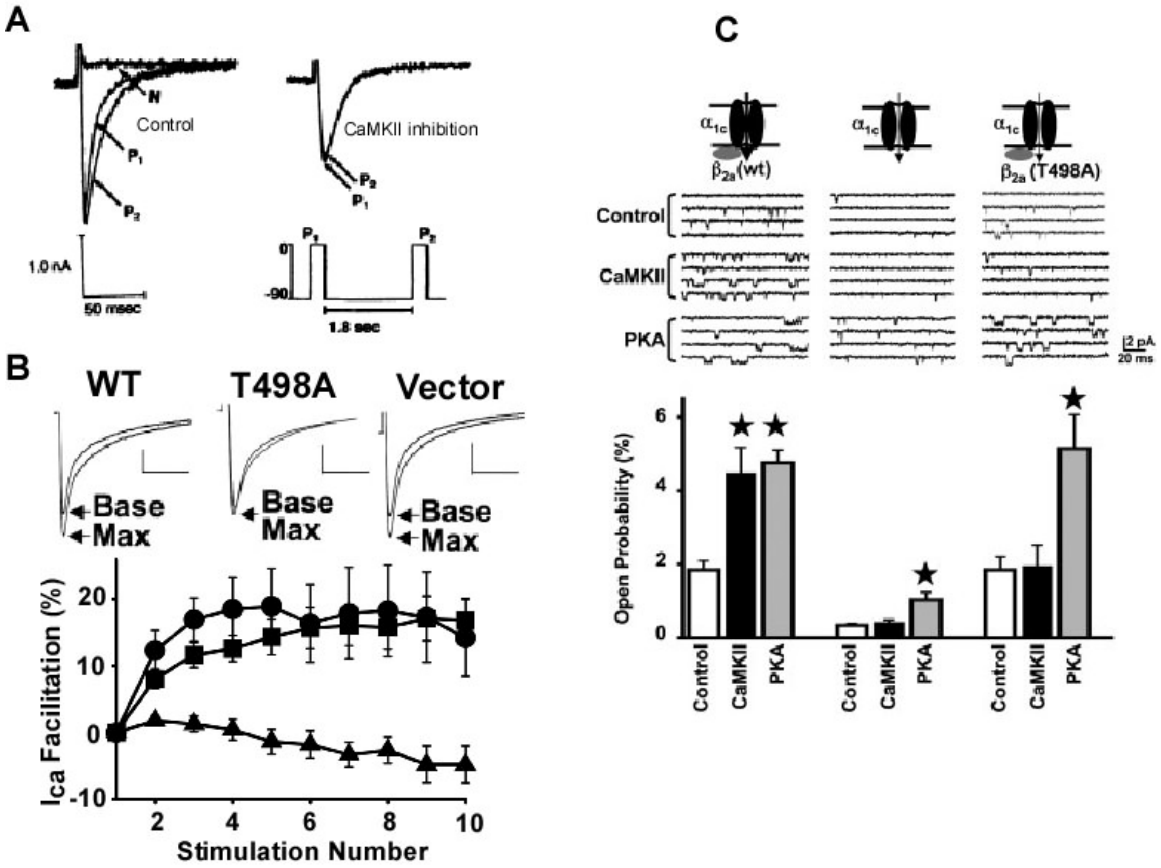

Figure 4. Common CaMKII-dependent forms of LTCC facilitation.
(**A**) I_{ca} at two successive pulses P_1 and P_2. The first pulse results in an increased I_{ca} peak and prolonged decay of I_{Ca} following the second pulse, a process known as facilitation. Nifedipine (N), a selective L-type Ca^{2+}-channel blocker blocks the currents. A peptide that inhibits CaMKII activation blocks facilitation and other CaM-dependent processes. *Adapted from [63, 140]*

(**B**) Repetitive depolarization protocols (0.5 Hz) revealed normal facilitation in cardiomyocytes transduced with control (●) or FLAG-β_{2a}(wt) (■) lentivirus, but not in cells transduced with FLAG-β_{2a}(T498A) lentivirus (▲). Representative current traces are shown above with horizontal and vertical scale bars representing 50 ms and 2 pA/pF, respectively. *Adapted from [79].*

(**C**)Thr498 in the β_{2a} subunit mediates regulation of recombinant LTCCs. Cartoons at the top show LTCCs containing $Ca_v1.2\alpha_1$(α_{1c}) and β_{2a}(wt), $Ca_v1.2\alpha_1$ alone, or $Ca_v1.2\alpha_1$ and β_{2a}(T498A) expressed in tsA201 cells. Representative sweeps of single channel activity are shown after incubation of cytosolic faces of excised membrane patches without or with constitutively active CaMKII or PKA. The bar graph shows cumulative open probabilities (P_o) for channels under each condition. *Adapted from [79].*

BAPTA[140]. Additional studies showed that LTCCs and CaMKII closely coexist within microdomains at the plasma membrane such as neuronal dendritic spines[33, 34, 38] and cardiomyocyte Z-lines[79, 97, 146]. The close proximity of CaMKII and LTCC is also reflected in their physical association and coimmunoprecipitation from heart [63, 147].

Role of the α_1 subunit in CaMKII-dependent facilitation

LTCCs were identified as a CaMKII substrates nearly two decades ago[139] but only recently have specific sites been linked to function. CaMKII phosphorylates Ser 1512 and Ser1570 at the C-terminus of the α_1 subunit to induce VDF[63]. CaMKII also phosphorylates Ser439 in the Timothy syndrome $Ca_v1.2\alpha_1$ mutant, to increase channel activity under basal conditions[61]. Similarly, CaMKII-dependent phosphorylation at Thr1604 of $Ca_v1.2$ slows the rundown of Ca^{2+} currents in cardiomyocytes[70]. CaMKII potentiates $Ca_v1.3$ LTCCs via CaMKII phosphorylation at Ser1486 in the $Ca_v1.3\alpha_1$ EF hand[38].

Direct binding to and/or phosphorylation of the α_1 subunit by CaMKII supports Ca^{2+} and voltage-dependent facilitation of LTCCs induced by multiple paradigms in heterologous cells coexpressing β_1 or β_2 subunits[61, 63, 147]. Recent studies showed that CaMKII binds to multiple sites on the $Ca_v1.2\alpha_1$ subunits and coimmunoprecipitates with the α_1 subunit[63, 147]. Mutation of one of these CaMKII binding sites on the α_1 subunit abolishes LTCC facilitation in frog oocytes[147]. However, multiple factors confound this proposed

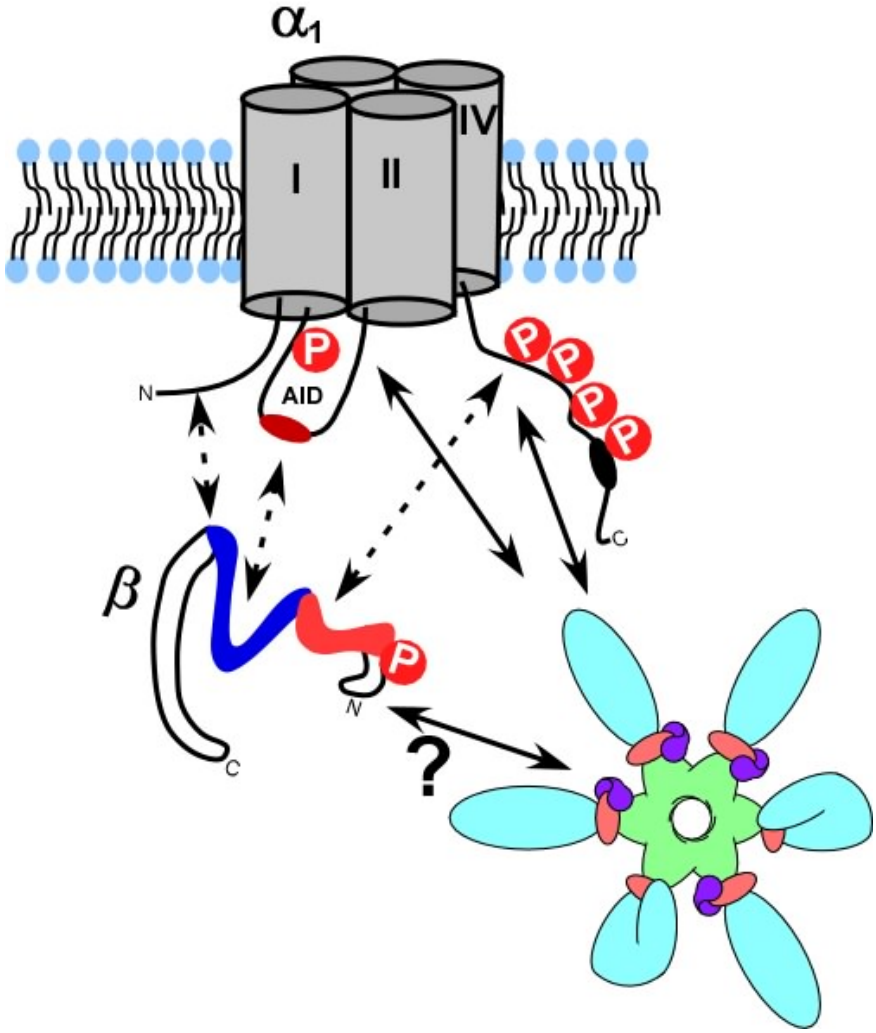

Figure 5. A model for β subunit-dependent facilitation of LTCCs by CaMKII before the findings in this thesis.
Arrows indicate binding. Multiple interactions with α_1 subunit target β subunits (*dashed arrows*) to LTCCs. Activated CaMKII binds α_1 at multiple domains (*solid arrows*). Increased local CaMKII concentrations enhances phosphorylation of α_1 at S1517 or S1575 (or S439 in the Timothy Syndrome mutant) and β_{2a} at T498, resulting in LTCC facilitation. My studies test if the β_{2a} subunit is responsible for targeting CaMKII to LTCC complexes (**?**), investigate the role of β_{2a} in modulating phosphorylation of the α_1 and β subunit subunit, and begin to investigate the dynamic assembly of these complexes.

α_1-dependent mechanism. First, the α_1 subunit was mutated within the C-terminal IQ domain[58] , which could interfere with CaM binding. Secondly, while mutation of the (TVGKFY) domain disrupts CaMKII binding to a purified C-terminal fragment of $Ca_v1.2\alpha_1$, it does not affect CaMKII binding to full-length α_1 [147]. Thirdly, the relevance of the LTCC regulation in oocytes to cardiomyocytes or neurons is unclear. Our lab has focused on the role of the β subunit in LTCC regulation by CaMKII.

Role of β subunits in CaMKII-dependent LTCC regulation

LTCC β subunits contain structural features that enable MAGUK family of scaffolding proteins to cluster proteins into signaling units and to link them to the cytoskeleton and intracellular signaling pathways. A role of the β subunits in LTCC facilitation has also been demonstrated (**Figure 4**) [29, 56, 79, 148, 149]. CaMKII efficiently phosphorylates all four LTCC β subunit isoforms and phosphorylation of Thr498 of the β_{2a} is critical for CaMKII-dependent facilitation of single LTCC opening and LTCC facilitation in cardiomyocytes (**Figure 4B and 4C**) [79, 150]. In addition to phosphorylating the β subunits of LTCCs, CaMKII appears to associate with them. Endogenous CaMKII and β subunits are localized along z lines of cardiomyocytes and CaMKII punctae colocalize with exogenous β_{2a} at cardiomyocyte tubules[79]. Interestingly, we note that amino acids 485-505 of β_{2a} (containing the Thr498 phosphorylation site) are very similar to the CaMKII autoinhibitory domain and the CaMKII binding domain of NR2B, suggesting that CaMKII may actually bind the LTCC β subunit (**Figure 6**).

Notably, phosphorylation sites in the autoregulatory domain (T286/7) and in NR2B (Ser1303) are conserved with T498 in β_{1b} and β_{2a} but not in β_3 and β_4, suggesting the interaction is isoform selective and dynamically regulated (**Figure 6)**

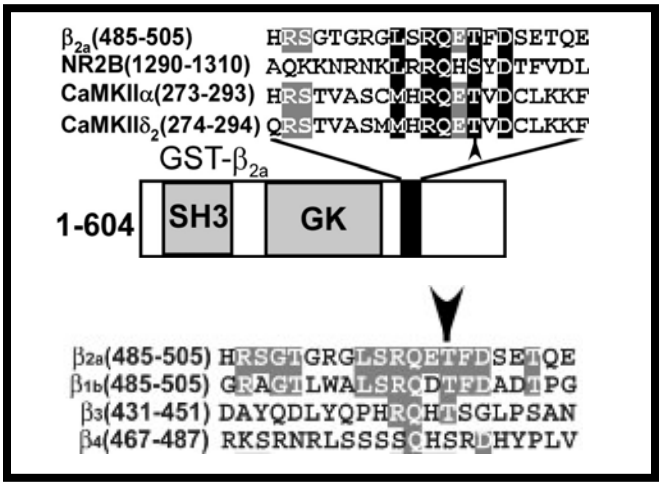

Figure 6. Similarity between NR2B, CaMKII regulatory domain and the region surrounding Thr498 site in β_{2a}.
*Top.*Schematic structure of β_{2a}. Amino acids 485-505 of β_{2a} contain a CaMKII binding motif that is conserved in NR2B and CaMKII isoforms. Phosphorylation sites are indicated by the arrowhead. *Residues conserved in all (black) or in some (grey) of these domains are highlighted. Bottom*. The amino acid sequence of the putative CaMKII binding domain in β_{2a} is aligned with similar sequences from other β isoforms: identical residues are shown in gray boxes.

1.9 HYPOTHESIS

Based on the above background information, I hypothesized that β subunits of LTCCs differentially and dynamically target CaMKII to modulate the phosphorylation of LTCC subunits.

1.10 SPECIFIC AIMS

AIM 1 (Chapter 2) addresses the molecular mechanisms of dynamic assembly and regulation of binary LTCC β-CaMKII complexes. *The specific aims are to:*

a. Test CaMKII interaction with β_{2a}* and explore the molecular basis for the interaction *in vitro.

b. Determine the effect of phosphorylating Thr498 of β_{2a} on its interaction with CaMKII.

c. Examine the regulation β_{2a}-CaMKII association by Thr498 phosphorylation in heterologous cells.

AIM 2 (Chapter 3) determines if the β-CaMKII interactions in **AIM 1** enable targeting of CaMKII to phosphorylate LTCC subunits. *The specific aims are to:*

a. Test if the LTCCα_1, β and CaMKII are associated in brain.

b. Determine the role of β subunits in targeting CaMKII to LTCCs in vitro.

c. Determine the role of β_{2a} in targeting of CaMKII to LTCCs in HEK cells.

d. Determine the effect of CaMKII binding on LTCC phosphorylation.

In **Chapter 4,** the general implications of the findings in my studies are discussed and summarized.

CHAPTER II

THE MOLECULAR BASIS AND DYNAMIC REGULATION OF CAMKII INTERACTION WITH β_{2A}

2.1 CHAPTER SUMMARY

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylates the β_{2a} subunit of voltage-gated Ca^{2+} channels at Thr498 to facilitate cardiac L-type Ca^{2+} channels. CaMKII colocalizes with β_{2a} in cardiomyocytes and a domain in β_{2a} that contains Thr498 exhibits an amino acid sequence similarity to the CaMKII autoinhibitory domain and to a CaMKII binding domain in the NMDA receptor NR2B subunit. Here I show that activated/Thr286-autophosphorylated CaMKII binds LTCC β_{2a} with a high affinity (~89nM) comparable that of NR2B subunits and associates with the LTCC β_{2a} in HEK293 cells. I also explored the selectivity of the binding of CaMKII to Ca^{2+} channel β subunit isoforms. Activated/autophosphorylated CaMKII binds to β_{1b} and β_{2a} with a similar apparent affinity but does not bind to β_3 or β_4. Residues surrounding Thr498 in β_{2a} are highly conserved in β_{1b} but are different in β_3 and β_4. Mutagenesis of Leu493 to Ala substantially reduces CaMKII binding *in vitro* and in intact cells but does not interfere with β_{2a} phosphorylation at Thr498. Site-directed mutagenesis of this domain in β_{2a} showed that Thr498 phosphorylation promotes dissociation of CaMKII-β_{2a} complexes *in vitro* and reduces interactions of CaMKII with β_{2a} in cells. Prephosphorylation of β_{1b} and β_{2a} by CaMKII substantially reduces the binding of autophosphorylated CaMKII. In combination, these data show that

phosphorylation dynamically regulates the interactions of specific isoforms of the Ca^{2+} channel β subunits with CaMKII.

2.2 MATERIALS AND METHODS

Generation of Plasmid Constructs

The open reading frames of the rat β_{1b}, β_{2a}, β_3 and β_4 (Accession Numbers X61394, M80545.1, M88751 and L02315 respectively), generous gifts from Dr. Timothy Kamp and Dr. Ed Perez-Reyes, were amplified by PCR and ligated into pGEX-4T1 (Amersham Pharmacia Biotech) by Dr. Rong Zang and Yunji Wu. The β_{2a} subunit was also subcloned into pFLAG-CMV-2 (Sigma-Aldrich), pIRES (Clontech), and pLenti (Invitrogen). Murine CaMKIIα and rat CaMKIIδ coding sequences were inserted into pcDNA3. The cDNAs encoding β_{2a} were mutated by Dr. Chad E. Grueter essentially as described in the QuikChange kit (Stratagene). The pcDNA plasmid encoding a constitutively active T287D mutation of myc-CaMKIIδ_2 was a generous gift from Dr. E. Olson (UTSW, Dallas).

GST Fusion Protein Expression and Purification

GST fusion proteins were expressed and purified as described in [79]. Protein concentrations were determined by Bradford assay (BioRad) using bovine serum albumin as the standard and were confirmed by resolving proteins on SDS-polyacrylamide gels followed by Coomassie-Blue staining.

CaMKII Purification and Autophosphorylation

Recombinant rat CaMKIIδ_2 or mouse CaMKIIα purified from baculovirus infected Sf9 insect cells were autophosphorylated at Thr287 or Thr286, respectively, using ATP or [γ-^{32}P]ATP, essentially as described previously [151].

CaMKII Plate Binding Assays

GST fusion proteins in 0.2 mL of plate-binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1% (v/v) Tween-20, 5 mg/mL bovine serum albumin) were incubated for 18−24 h at 4 °C in glutathione-coated wells. After 3 washes with buffer, wells were incubated at 4 °C with the indicated concentrations of ^{32}P-labeled, Thr286/7 autophosphorylated CaMKIIα or CaMKIIδ_2 (0.2 mL) for 2 h and then washed (8 times, 0.2 mL of ice-cold buffer). The bound kinase was quantified using a scintillation counter. To monitor dissociation of preformed CaMKII-β_{2a} complexes, GST-β_{2a} (wild-type or T498A: ≈5 pmol) was immobilized in glutathione-coated multi-well plates (Pierce, Rockford, IL) and then incubated for 2 h at 4 °C with [^{32}P-T286]CaMKIIα (0.25 μM) in binding buffer. Wells were rinsed 8 times in binding buffer, and immobilized complexes were then incubated with 50 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.25 mg/mL bovine serum albumin, 0.1% Triton X-100, 1 mM dithiothreitol, 10 mM magnesium acetate, with or without 0.5 mM ATP. Soluble/dissociated CaMKII was removed from the wells at the indicated times and quantified by scintillation counting.

CaMKII Gel Overlays

GST fusion proteins (50 pmol) were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Approximately equal protein loading was confirmed by staining membranes using Ponceau-S. Membranes were blocked and then incubated for 2 h at 4 °C with ^{32}P-labeled, Thr286/7 autophosphorylated CaMKIIα or CaMKIIδ_2 (100 nM), essentially as described previously [152]. After washing, bound CaMKII was quantified using a phosphoimager.

CaMKII Phosphorylation of GST-β Subunits

Purified GST-β subunits (or GST alone as a blank) were incubated at 30°C in 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mg/mLbovine serum albumin, 1 mM dithiothreitol, 0.4 mM [γ-^{32}P]ATP (≈500 cpm/pmol) or 0.4 mM ATP containing purified CaMKII. After 20 minutes phoshorylation was stopped by denaturing the proteins in SDS-PAGE loading buffer.

Immunoblotting

Samples were resolved on Tris-glycine SDS-polyacrylamide gels and transferred to nitrocellulose membranes by wet transfer in 10 mM CAPs, pH 7.5 and 10% methanol at 4^{o}C. Membranes were blocked in 5% (w/v) milk in TTBS (50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, 0.1% (v/v) Tween-20) and then incubated with primary antibodies overnight at 4°C. After being washed 6 times for >5 min each, membranes were incubated for 1 h at room temperature with horseradish

peroxidase conjugated secondary antibody. The washed membranes were developed using enhanced chemiluminescence.

Co-immunoprecipitations from HEK293 Cells

Experiments were performed as described in [79]. Briefly, HEK293FT cells were transfected with FLAG-β_{2a} (wild-type, T498A, T498E, or L493A), CaMKIIα (or myc-CaMKIIδ_2(T287D), and/or the FLAG vector alone. Cell lysates were immunoprecipitated with anti-FLAG-coated agarose beads (40 µL, Sigma) and then immunoblotted.

Statistics

Data were expressed as mean ± SEM. Paired comparisons were performed using the Student's t test. Multiple group comparisons were performed using one-way or two-way ANOVA with Bonferoni post-hoc testing, unless otherwise noted. The null hypothesis was rejected if $p < 0.05$.

2.3 RESULTS

Activated CaMKII binds LTCC β_{2a} with high affinity

As a first step toward determining whether LTCC β subunits play a role in CaMKII phosphorylation-mediated facilitation of cardiac LTCCs, I performed CaMKII overlay and glutathione agarose CaMKII cosedimentation assays with a glutathione S-transferase (GST) fusion protein containing the entire sequence of the rat β_{2a} subunit (GST-β_{2a}[wt]). A GST-NR2B (1260-1339S1303A) protein that binds activated CaMKII (**see Introduction**) was used as a positive control. The major neuronal and cardiac isoforms of CaMKII (CaMKIIα and CaMKIIδ respectively) associated with GST-β_{2a}(wt) (**Figure 7A and 7B**). Binding required prior activation of CaMKII by Ca^{2+}/CaM binding in the presence of nucleotide or autophosphorylation in the regulatory domain (Thr287 in CaMKIIδ or Thr286 in CaMKIIα, **Figure 7B and 7C**). In a glutathione-plate binding assay, GST-β_{2a}(wt) bound activated CaMKIIδ with an apparent K_d 90 nM (**Figure 7D**), more than an order of magnitude lower than estimated levels of CaMKII in heart[146, 152]. Because of the apparent similarity of CaMKIIα and δ isoforms in binding to the LTCC β_{2a}, and due to our initial focus on cardiac LTCCs, I did several of the initial binding studies in this chapter using the CaMKIIδ_2 isoform. The CaMKII isoform used in each experiment is specified in the figure legend.

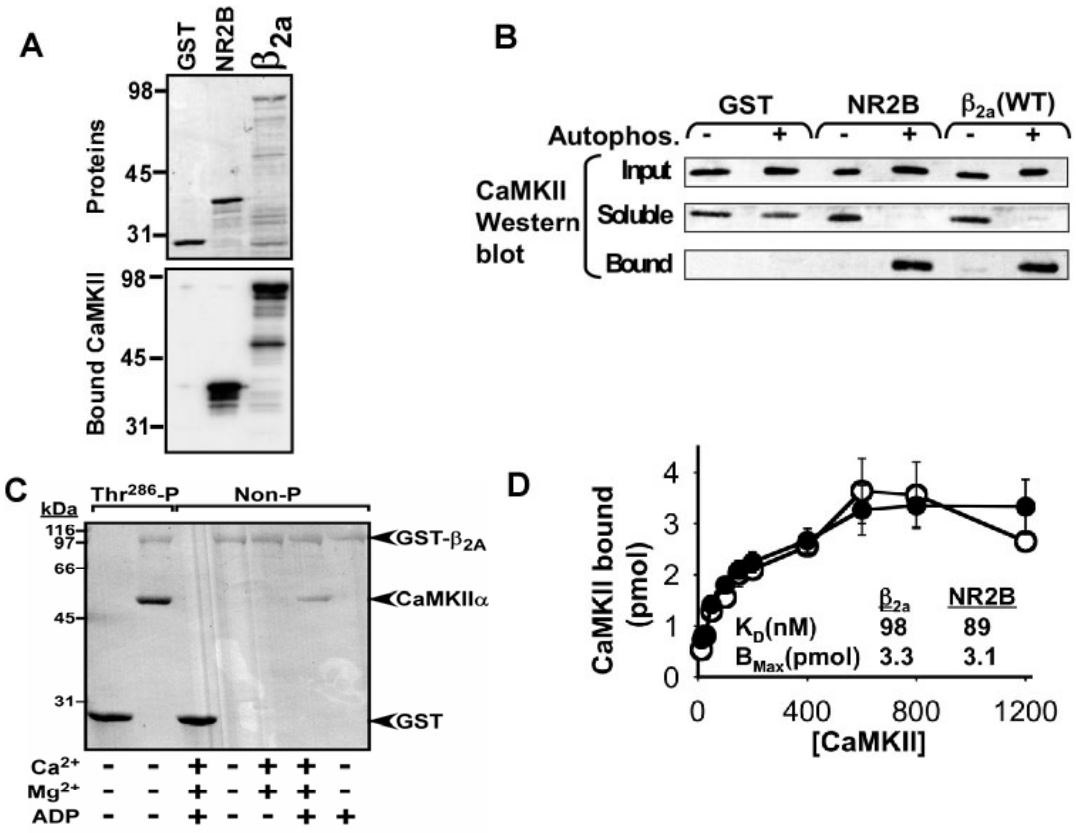

Figure 7. Binding of activated CaMKII to β_{2a}

(**A**) Thr286 autophosphorylated CaMKIIα binding to GST-β_{2a}(wt) and GST-NR2B using a CaMKII overlay assay. n =1 using CaMKIIα and >3 using CaMKIIδ_2 (data not shown)

(**B**) Thr287 autophosphorylation-dependent binding of CaMKIIδ_2 to GST-β_{2a}(wt) and GST-NR2B using a cosedimentation assay. The input, soluble, and bound fractions were analyzed by immunoblotting for CaMKII. Similar results were obtained with CaMKIIα. n>3

(**C**) Ca^{2+}/CaM-dependent Binding of β_{2a} to CaMKIIα using a glutathione agarose cosedimentation assay. First two lanes: GST or GST-β_{2a} was incubated with Thr286-autophosphorylated CaMKIIα. Remaining lanes: GST or GST-β_{2a} was incubated with non-phosphorylated CaMKII in the presence or absence of Ca^{2+}, Mg^{2+} and ADP as indicated. Bound proteins were separated by SDS and transferred to nitrocellulose and stained with Ponceau-S. The data is representative of at least 2 experiments. *Data shown is from an experiment performed by Dr. AJ. Robison.*

(**D**) CaMKIIδ_2 binds β_{2a} (●) and NR2B (○) with similar affinity in a glutathione plate binding assay. The inset table reports equilibrium binding parameters (apparent K_D and B_{max}). Data is displayed as mean ± SEM, n = 3.

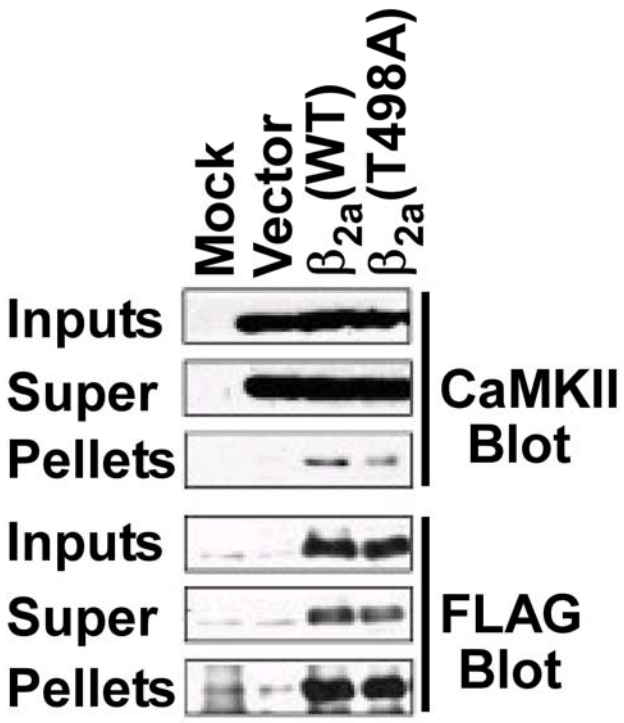

Figure 8. CaMKII coimmunoprecipitates with FLAG-β_{2a}.
HEK293 cells were transiently transfected to express CaMKIIα with either FLAG-β_{2a}(wt) or FLAG-β_{2a}(T498A). Control cells were mock transfected or expressed CaMKII alone. Cell lysates were immunoprecipitated by using anti-FLAG agarose beads, and aliquots of inputs, supernatants (super), and immune pellets were immunoblotted for CaMKII (top) and FLAG proteins (bottom). *Experiment performed by Dr. Chad E. Grueter.*

Coimmunoprecipitation of CaMKII and β_{2a} from HEK cells

To investigate whether CaMKII associates with β_{2a} subunits in intact cells, CaMKII was coexpressed with FLAG-tagged β_{2a} proteins in HEK293 cells. Immunoprecipitations using FLAG antibodies resulted in the coprecipitation of CaMKII from cell lysates containing FLAG-β_{2a}(wt), but not from lysates that did not contain FLAG proteins (**Figure 8**). Taken together, these findings identify the β_{2a} subunit as a *bona fide* CaMKII binding protein and suggest that β_{2a} is a CaMKII-associated protein (CaMKAP) *in situ*.

Mapping the CaMKII binding domain on LTCC β_{2a}

In order to identify the relationship of the CaMKII binding site to conserved domains in the β_{2a} subunit, we screened a library of GST-fusion proteins containing various fragments of β_{2a} by using gel overlay assays. CaMKII bound to all fragments that contained residues 410–505 of β_{2a}, but not to fragments that lacked this region (**Figure 9A**). The structure of SH3/GK domains reveals insights into the mechanism for constitutive association of the α_1 and β subunits (**see Introduction**), but the domain containing residues 410–505 was not resolved in these structures. Inspection of the primary amino acid sequence of the CaMKII binding domain revealed that residues 486–500 are homologous to a portion of the CaMKII autoregulatory domain (residues 274–289 in CaMKIIα) and to a well-established CaMKII binding domain (residues 1298–1305) in the NR2B subunit of the NMDA receptor (**Figure 9B and Figure 6**) [153-155].

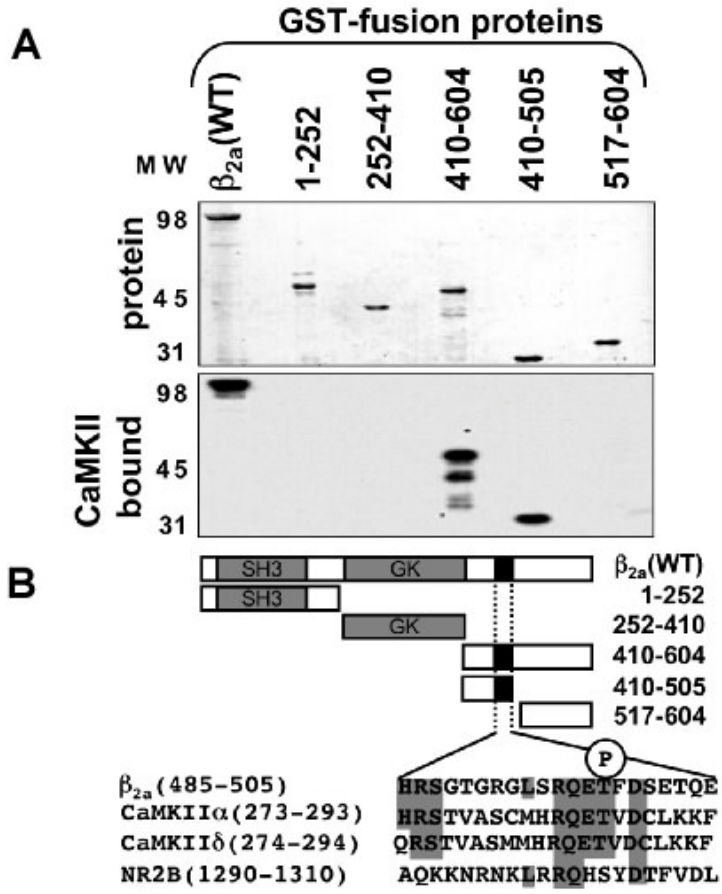

Figure 9. Mapping the CaMKII binding domain on β_{2a}
(**A**) Mapping the CaMKIIδ_2 binding domain to amino acids 410–505 using gel overlay assays.

(**B**) β_{2a} domain map. SH3- and GK-homology domains are indicated in gray, and the CaMKII binding domain is indicated in black. Partial amino acid sequences of β_{2a}, CaMKIIα, CaMKIIδ, and NR2B are aligned below with sequence identities in grey boxes.

The binding parameters for CaMKII interaction with GST-β_{2a}(wt) were very similar to those for CaMKII interaction with GST-NR2B(1260–1339) (**Figures 7D**).

Selective interactions of CaMKII with VGCC β subunits *in vitro*.

Thr498 of the β_{2a} variant lies within a CaMKII-binding domain that is C-terminal to the SH3 and GK domains (**Figure 9**). Alignment of the amino acid sequence of the domain surrounding Thr498 in β_{2a} with other β subunit isoforms revealed variable conservation (**Figure 10A**). A CaMKII consensus phosphorylation motif LXRXXS/T is present in both β_{2a} and β_{1b}, and there is additional amino acid sequence similarity outside this motif, but key residues from this motif are missing in β_3 and β_4. On the basis of these alignments, we hypothesized that CaMKII would bind to β_{1b} in a similar manner to its interaction with β_{2a} but not to β_3 or β_4.

To test this hypothesis, I determined the binding of nonautophosphorylated and Thr287-autophosphorylated CaMKIIδ to GST-tagged β subunit isoforms. Autophosphorylated CaMKII bound to GST-β_{1b} and GST-β_{2a} but not to GST-β_3 and GST-β_4 in glutathione agarose co-sedimentation assays but there was no significant binding of non-phosphorylated CaMKII to any GST-β subunit isoforms (**Figure 10B**). To quantify CaMKII binding to β isoforms, I immobilized GST-β proteins in glutathione-coated 96-well plates and then incubated them with various concentrations of purified ^{32}P-autophosphorylated CaMKII. The binding of CaMKII to GST-β_{1b} was indistinguishable from the binding to GST-β_{2a}, but

binding to GST-β_3 and GST-β_4 was <1% of the binding to GST-β_{2a} (**Figure 10C**). Binding of Thr286-autophosphorylated CaMKII to both GST-β_{1b} and GST-β_{2a} was concentration-dependent and saturable (**Figure 10D**). GST-β_{1b} exhibited a significantly higher apparent affinity for CaMKII than did GST-β_{2a} (apparent K_d values of 35 ± 12 and 120 ± 21 nM CaMKII subunit, respectively, n = 4, $p < 0.01$). GST-β_3 and GST-β_4 failed to bind significant amounts of the kinase even at a concentration of 1200 nM CaMKII subunit (data not shown). Thus, CaMKII does not appear to bind significantly to the β_3 and β_4 isoforms but interacts with the β_{1b} isoform with an ≈3-fold higher affinity than with β_{2a} following activation by Thr286/7 autophosphorylation.

Interaction of β subunits with CaMKII is modulated by phosphorylation *in vitro*.

Thr498 and several additional serine and threonine residues lie within the CaMKII binding domain of β_{2a}, suggesting that the interaction with CaMKII may be modulated by phosphorylation. Therefore, GST-β isoforms were preincubated with activated CaMKII in the presence or absence of ATP and then separated from reaction components by SDS-PAGE. The electrophoretic mobility of each GST-β isoform was reduced following preincubation with ATP and CaMKII (**Figure 11A: protein stain**), consistent with the relatively high phosphorylation stoichiometry (6-12 mol/mol) under these conditions[79, 150]. An overlay assay was then used to assess the binding of ^{32}P-autophosphorylated CaMKII. Non-

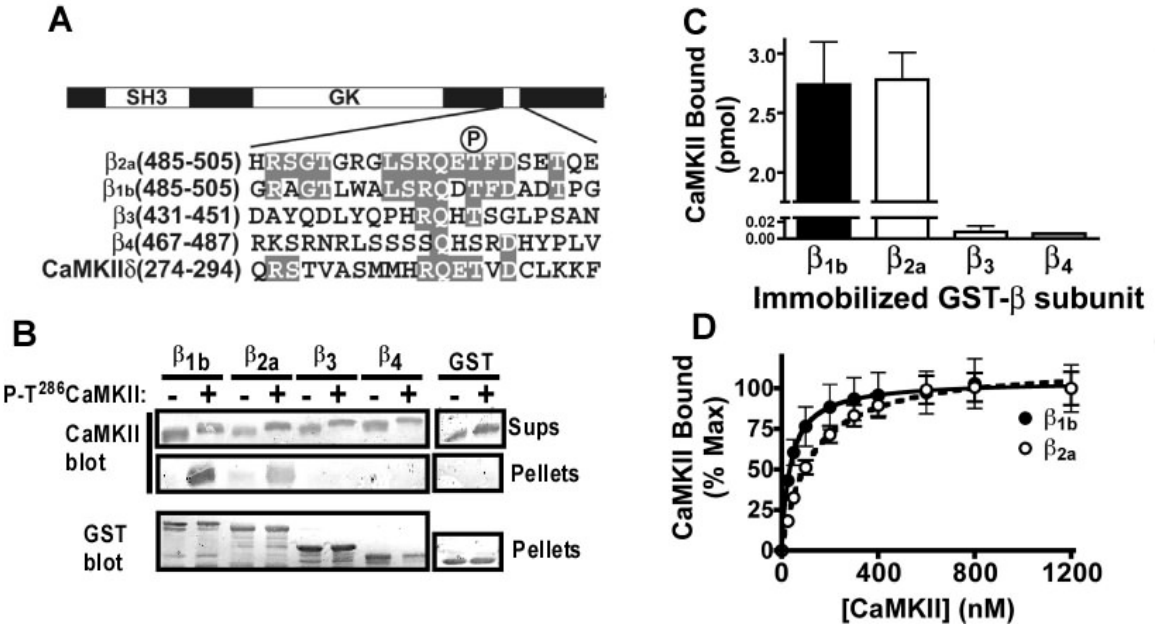

Figure 10. Differential CaMKII binding to VGCC β subunit isoforms.
(**A**) Schematic domain structure of the β_{2a} subunit showing SH3 and guanylate kinase-like (GK) domains, with the C-terminal CaMKII-binding domain containing the Thr498 phosphorylation site (indicated by the "P"). The amino acid sequence of the CaMKII binding domain in β_{2a} is aligned with similar sequences from other β isoforms and the CaMKIIδ autoregulatory domain (surrounding Thr287): identical residues are shown in gray boxes.

(**B**) CaMKIIα binding to GST-β isoforms in a glutathione-agarose cosedimentation assay. GST-β subunit isoforms or GST alone (50 pmol) and either nonphosphorylated or Thr286 autophosphorylated CaMKIIα (100 pmol subunit) were incubated with glutathione agarose. Aliquots of the supernatant (sups) and the beads were analyzed by immunoblotting as indicated.

(**C**) GST-β subunit isoforms were immobilized in glutathione-coated multi-well plates (100 pmol/well) and incubated with ^{32}P-labeled Thr287 autophosphorylated CaMKIIδ$_2$ (50 nM subunit).

(**D**) Concentration-dependent binding of Thr287 autophosphorylated CaMKIIδ$_2$ to GST-β_{2a} (○) and GST-β_{1b} (●) in glutathione-coated multi-well plates. Both panels B and C plot binding as mean ± SEM from four observations (β_{2a} and β_{1b}) or the mean of two observations (β_3 and β_4)

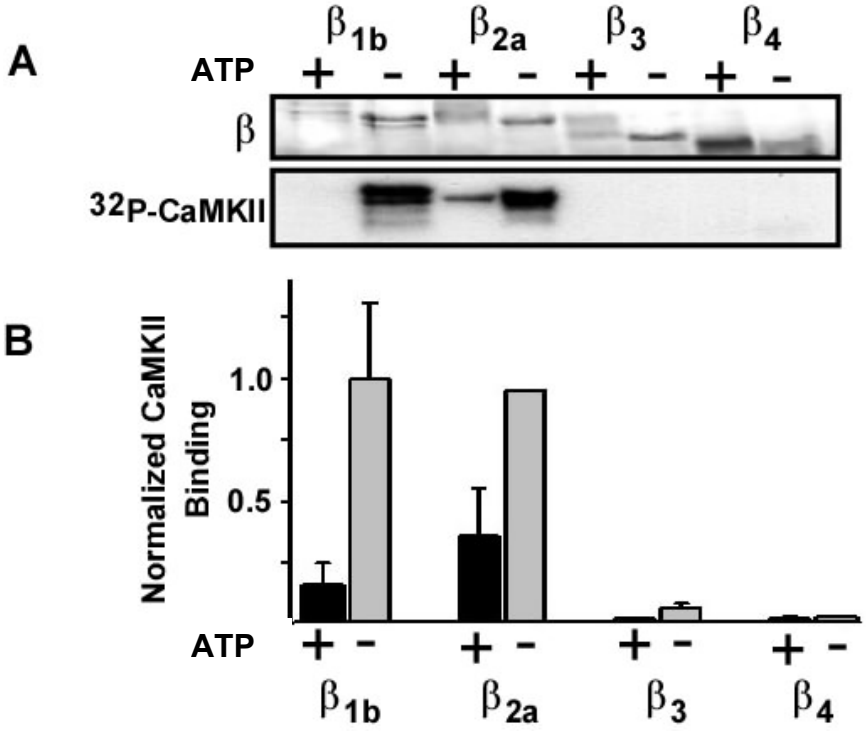

Figure 11. Effect of phosphorylation on CaMKII binding to β isoforms. (**A**). Prephosphorylation of GST-β_{2a} and GST-β_{1b} inhibits CaMKII binding. GST-β isoforms were preincubated with activated CaMKIIδ_2 in the presence or absence of ATP, as indicated. Reactions were resolved by SDS-PAGE and transferred to a nitrocellulose membrane for overlay with ^{32}P-labeled Thr287 autophosphorylated CaMKIIδ_2. The top panel shows a protein stain of the membrane prior to overlay and a representative autoradiograph to detect bound CaMKII is shown below.

(**B**). Binding was quantified using a phosphoimager and normalized to the binding detected using non-phosphorylated GST-β_{2a}: the mean ± SEM from three experiments is plotted. Data were analyzed by 2-way ANOVA: *: $p < 0.001$ vs binding to non-phosphorylated GST-β_{2a} and #: $p < 0.05$ vs binding to the corresponding non-phosphorylated protein. *These experiments were performed in collaboration with Dr. Chad Grueter.*

phosphorylated GST-β_{1b} and GST-β_{2a} proteins bound substantial, and comparable, amounts of CaMKII (**Figure 11A and B**). Pre-phosphorylation significantly reduced CaMKII binding to both proteins by 70−80% (**Figure 11A and B**). No significant interactions were seen between CaMKII and either GST-β_3 or GST-β_4, whether or not these protein were pre-phosphorylated. In combination, these data suggest that phosphorylation of the β subunit by CaMKII modulates the binding of CaMKII to the β_{1b} and β_{2a} isoforms.

Mechanism of CaMKII Binding to β_{2a}

I began to explore the mechanism for a dynamically regulated CaMKII binding to β_{2a} by assessing the effect of mutating residues within the CaMKII binding domain of β_{2a} on the interaction with CaMKII. Mutation of Thr498 to Ala or Glu prevented or mimicked phosphorylation at this site, respectively. In addition, residues homologous to Leu493 at the p-5 position relative to Thr498 in β_{2a} and β_{1b} are conserved in other high affinity CaMKII phosphorylation sites that form stable complexes with the CaMKII catalytic domain prior to phosphorylation (e.g., Ser1303 in NR2B and Thr286/7 in CaMKIIα/δ). However, hydrophobic residues at the p-5 position are not conserved in β_3 or β_4 and are not generally considered to be part of the minimal consensus phosphorylation site. Therefore, we also mutated Leu493 to Ala. Binding of ^{32}P-autophosphorylated CaMKIIα to GST-β_{2a} in a CaMKII overlay was unaffected by T498A mutation but reduced by ~75% by a T498E mutation (**Figure 12A and B**). Similarly binding of CaMKII to GST-β_{2a} in glutathione coated multi-well plates was unaffected by the T498A mutation,

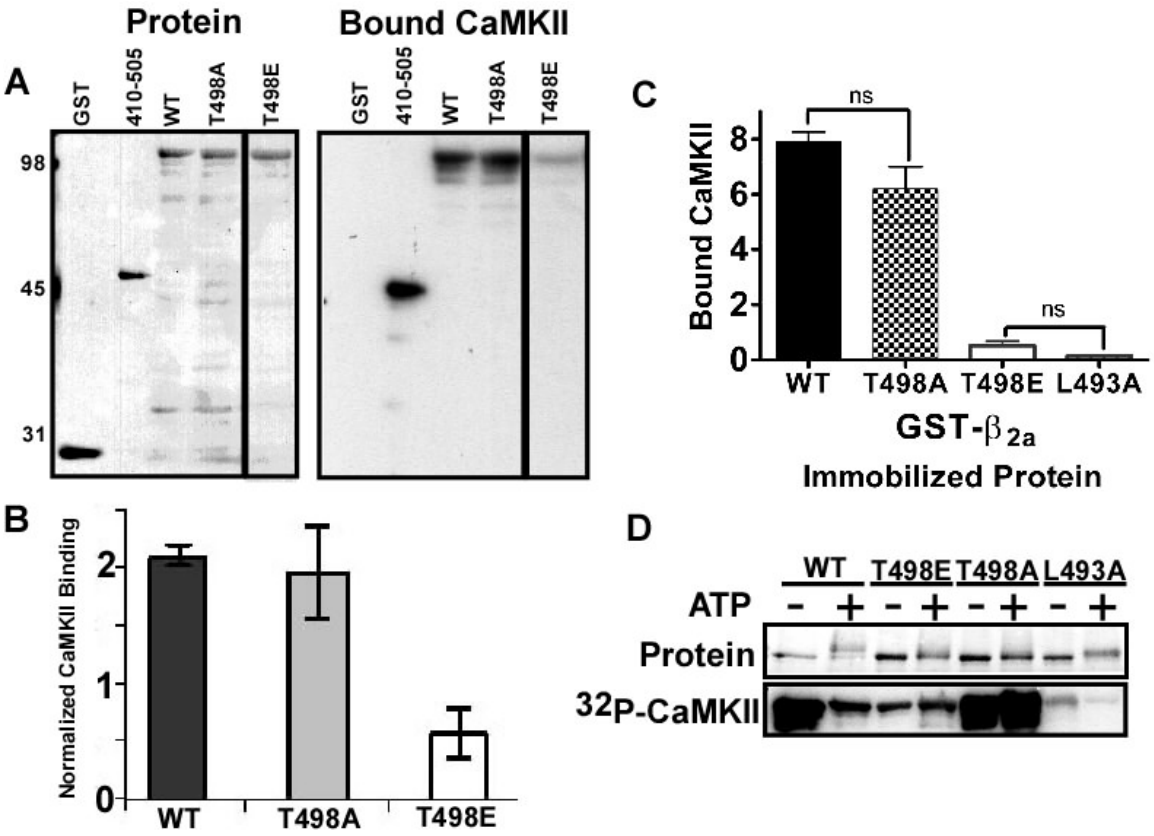

Figure 12. Identification of amino acids essential for CaMKII binding to β_{2a} and regulation of CaMKII binding to β_{2a} by Thr498 phosphorylation .
(**A**) GST-β_{2a} proteins were immobilized on nitrocellulose membranes and then overlayed with ^{32}P-labeled Thr287-autophosphorylated CaMKIIδ_2 (200 nM subunit).

(**B**) Quantitation of the binding in **A.** Data are mean +/- SEM of 3 experiments

(**C**) Purified GST-β_{2a} proteins (wild-type or with the indicated point mutations) or GST alone were immobilized in a glutathione-coated 96-well plate (100 pmol/well) and then incubated with ^{32}P-labeled Thr287-autophosphorylated CaMKIIδ_2 (100 nM subunit). After washing, bound CaMKII was quantified by scintillation counting. The data indicate the mean ± SEM (n = 3 experiments)

(**D**) Prephosphorylation of β_{2a} at Thr498 inhibits the association of CaMKII. Wild-type and mutated GST-β_{2a} proteins were preincubated with activated CaMKIIδ_2 in the presence or absence of ATP. Samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes to detect total protein loaded (top) and to probe with [^{32}P]CaMKIIδ_2 by overlay assay and autoradiography (bottom). *This experiment was performed by Dr. Chad Grueter*.

whereas the T498E and L493A mutations significantly reduced CaMKII binding by >90% (**Figure 12C**). These data demonstrate using multiple assays that the region surrounding Thr498 is critical for stable binding of Thr286-autophosphorylated CaMKII to the full-length β_{2a} isoform.

Phosphorylation of β_{2a} at Thr498 Disrupts CaMKII Binding

To closely examine the mechanism by which phosphorylation of β_{2a} by CaMKII interferes with binding of activated CaMKII, we pre-phosphorylated wild-type and mutated β_{2a} proteins and analyzed CaMKII binding using overlay assays. As determined in the glutathione plate-binding assay, WT and Thr498Ala-β_{2a} bound comparable amounts of activated CaMKII. Pre-phosphorylation of Thr498Ala-β_{2a} had no significant effect on binding of activated CaMKII, whereas pre-phosphorylation of wild-type β_{2a} significantly reduced binding by ≈80% in these assays (**Figure 12D**). As observed previously, the Thr498Glu mutation reduced CaMKII binding by ≈80%; phosphorylation by CaMKII had no significant additional effect on binding to T498E-β_{2a}. Interestingly, the Leu493Ala mutation significantly reduced binding by ≈90% in these assays, and pre-phosphorylation by CaMKII resulted in an additional significant decrease in binding (**Figure 12D**). Taken together, these data suggest that pre-phosphorylation of β_{2a} at Thr498 substantially reduces its association with CaMKII.

CaMKII Interaction with β_{2a} is Regulated in Cells

I first determined whether Thr498 in β_{2a} is phosphorylated in intact cells. Lysates of HEK293 cells expressing FLAG-β_{2a} (wild-type or mutated) with a constitutively active CaMKIIδ_2 (T287D) mutant were immunoprecipitated using FLAG antibodies. Blotting the immune complexes with FLAG antibodies revealed a relatively consistent expression and immunoprecipitation of the β_{2a} proteins. The phospho-Thr286 CaMKIIα antibodies detected the β_{2a} wild-type and L493A mutant but not the T498A- or T498E-β_{2a} proteins (**Figure 13A**). These data show that Thr498 can be phosphorylated to a similar extent in wild-type β_{2a} and L493A-β_{2a} in intact cells by constitutively active CaMKIIδ_2.To investigate the regulation of CaMKII interaction with β_{2a} in intact cells by modification of Thr498 and the surrounding domain, lysates of HEK293 cells expressing constitutively active myc-tagged CaMKIIδ_2 without or with FLAG-tagged β_{2a} subunits (wild-type or mutated) were immunoprecipitated using FLAG antibodies. CaMKII was readily detected in immune complexes containing FLAG-tagged wild-type β_{2a} or T498A-β_{2a} but could not be detected in immune complexes formed by T498E-β_{2a} or L493A-β_{2a} (**Figure 13B**). These findings demonstrate that the domain surrounding Thr498 is critical for the association of CaMKII with β_{2a} in cells and suggest that phosphorylation at Thr498 diminishes CaMKII binding to β_{2a} in cells.

Effect of Thr498 phosphorylation on CaMKII dissociation from β_{2a}

I also investigated the role of Thr498 phosphorylation in the context of preformed CaMKII-β_{2a} complexes. Complexes of autonomously active Thr286

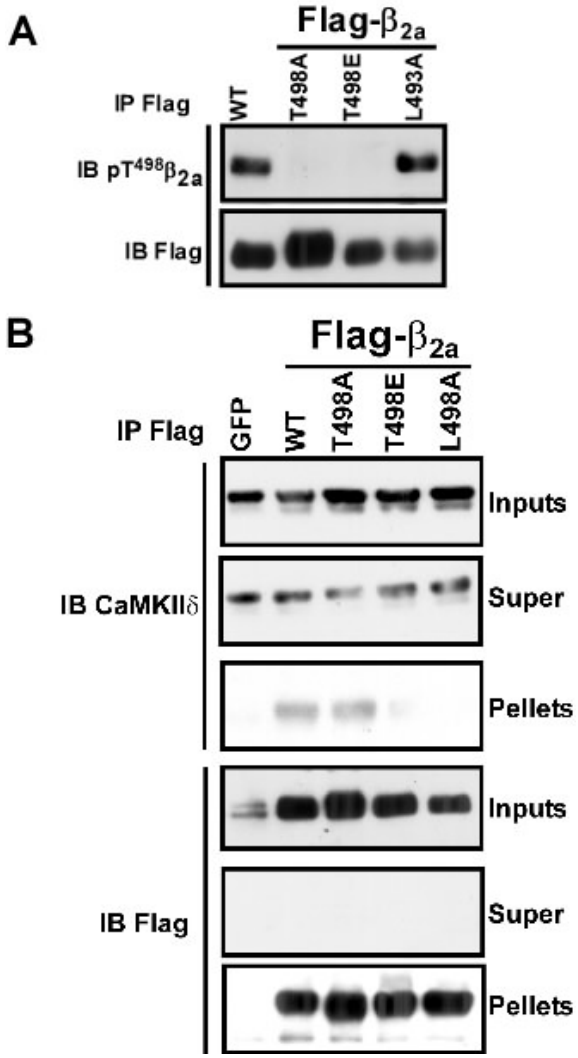

Figure 13. CaMKII interaction with β_{2a} is regulated by Thr498 phosphorylation *in situ*.
MYC-tagged CaMKIIδ_2 (T287D) was co-expressed with or without FLAG-tagged wild-type or mutated β_{2a} proteins in HEK293 cells. Aliquots of the cell lysates (inputs), FLAG immune supernatants (super), and FLAG immune complexes (pellets) were immunoblotted.

(**A**) Probing FLAG immune complexes using antibodies raised to phospho-Thr286 in CaMKIIα showed that wild-type and L493A β_{2a} are partially phosphorylated at Thr498 in intact cells.

(**B**) Probing FLAG immune complexes using CaMKIIδ antibodies revealed that CaMKII was associated with wild-type and T498A β_{2a} but could not be detected in the T498E and L493A β_{2a} immune complexes. The data are representative of >4 experiments.

autophosphorylated CaMKII bound to GST-β_{2a} (wild-type) were isolated in glutathione-coated multi-well plates and then incubated with or without the addition of ATP. The addition of ATP to these complexes induced phosphorylation at Thr498 and several other sites, as reflected by immunoblotting with phospho-Thr286 CaMKII antibodies and by a substantial reduction in the electrophoretic mobility of GST-β_{2a} (**Figure 14A**). In the absence of ATP, CaMKII complexes with wild-type β_{2a} were remarkably stable (<5% dissociation over a 2 h incubation), but the addition of ATP induced dissociation of ≈50% of bound CaMKII. The T498A mutation of β_{2a} had little effect on the stability of complexes with CaMKII in the absence of ATP but substantially reduced the rate and extent (by ≈20% in 2 h) of dissociation following addition of ATP as compared to wild-type β_{2a} (**Figure 14B and C**). The ATP-induced dissociation of CaMKII from preformed complexes with T498A-β_{2a} suggests that phosphorylation at other residues in β_{2a} and/or CaMKII may have a modest effect on the stability of these complexes, at least *in vitro*. In combination, these data suggest that phosphorylation of β_{2a} at Thr498 enhances the dissociation of CaMKII from preformed complexes with β_{2a} *in vitro*.

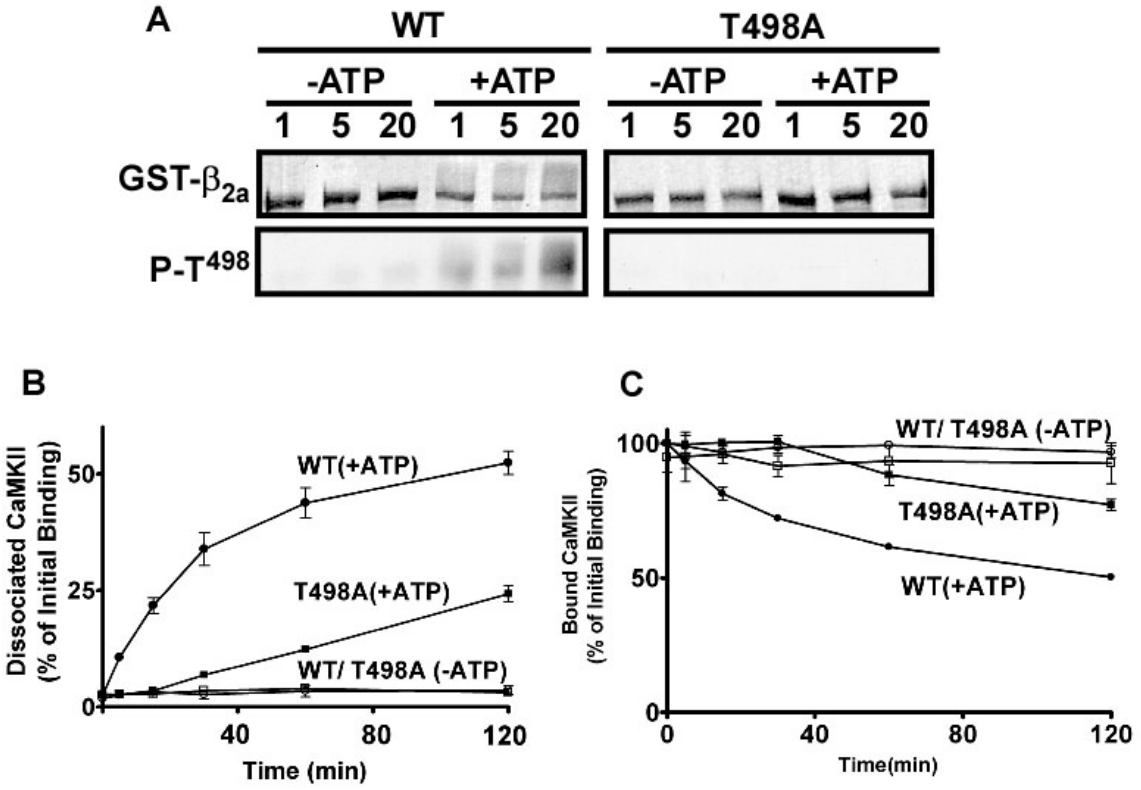

Figure 14. Phosphorylation of β_{2a} at Thr498 enhances dissociation of the CaMKII-β_{2a} complex.

(A) A preassembled complex of GST-β_{2a} (WT or T498A) and ^{32}P-T286 CaMKIIα was incubated with or without 0.5 mM ATP. Aliquots of resin were removed at the indicated times and cosedimented proteins were analyzed by western blotting for GST and phospho-T498.

(**B and C**)Complexes of GST-β_{2a} (wild-type or T498A) and [32P-T286]CaMKIIα in glutathione-coated multi-well plates were incubated with or without ATP in a dissociation buffer (see Materials and Methods). At the indicated times, dissociated [^{32}P-T^{286}]CaMKIIα was removed from the wells (**B**) or bound CaMKII (**C**) was quantified by scintillation counting. Data points represent mean ± SEM (n = 3) (n = 2 for β_{2a} T498A in the absence of ATP): error bars lie within symbols of some data points.

2.4 DISCUSSION

A diverse family of VGCCs regulates Ca^{2+} entry into excitable and non-excitable cells. The α_1 subunits confer core biophysical and pharmacological behavior of each type of VGCC. However, a cytosolic loop between the first and the second major transmembrane domains in the α_1 subunit is generally thought to constitutively interact with a β subunit. Alternative splicing of mRNAs from four mammalian genes generates >20 distinct β subunit proteins that have divergent roles in modulating the trafficking and biophysical properties of VGCCs[25, 156]. The β subunits share highly conserved SH3 and GK domains that form a compact structure, with a hydrophobic groove in the GK domain that interacts with the α_1 subunit[28]. However, some β subunits lack substantial parts of the SH3 and GK domains, yet still modulate LTCCs[25]. These observations support recent findings, showing that additional SH3-GK independent modulatory interactions between the α_1 and the β subunits are important for regulating VGCC activity [29, 157]. The variable domains presumably account for the unique effects of β subunit variants on the properties of α_1 subunits.

Most studies have focused on the roles of protein interactions and post-translational modifications of the α_1 subunit in modulating VGCCs. However, the importance of β subunits in regulating VGCCs has received increasing attention. Initial studies suggested that PKA phosphorylation of the β_2 subunit plays a role in facilitating LTCCs [77] although the importance of this modification in native cells has recently been questioned[65]. In addition, β subunits have been shown

to serve as scaffolding proteins that bind REM GTPases to inhibit VGCCs[82] and AHNAKs to link VGCCs to the actin cytoskeleton[158] . Our recent work showed that CaMKII colocalizes with β_{2a} in adult cardiomyocytes. Moreover, phosphorylation of β_{2a} at Thr498 is required for Ca^{2+}- and CaMKII-dependent facilitation of LTCCs in cardiomyocytes[79]. While β subunit variants have unique direct effects on the biophysical properties and trafficking of LTCCs, the β isoform selectivity of regulation by CaMKII and other modulators is poorly understood.

The β subunit variants tested here associate with multiple VGCC α_1 subunits (reviewed in [25]). Thus, the current results showing that activated/Thr286 autophosphorylated CaMKII forms stable complexes with β_{1b} and β_{2a}, but not with β_3 or β_4, lead to a hypothesis that the association of CaMKII with VGCC complexes will depend on the identity of the associated β subunit. The β_{1b} and β_{2a} subunits contain an LXRXXS/T motif similar to sequences surrounding phosphorylation sites in NR2B and the CaMKII autoinhibitory domain that also form stable complexes with the CaMKII catalytic domain. Mutation of Leu493 to Ala within the CaMKII binding motif reduced CaMKII binding to β_{2a} by >90% but surprisingly had little effect on the initial rate of phosphorylation at Thr498 or on the overall phosphorylation stoichiometry at multiple sites when measured *in vitro*[150]. The lack of conservation of this motif accounts for the failure to detect binding to β_3 or β_4.

Despite the apparent binding selectivity, CaMKII phosphorylates all four β subunit variants tested here with comparable relative rates and overall extent[150]. The high maximal phosphorylation stoichiometries[150] suggest that CaMKII efficiently phosphorylates several sites in each β isoform *in vitro*. Indeed, we previously identified six CaMKII phosphorylation sites in β_{2a} [79], and it will be important to identify phosphorylation sites in other β isoforms and to determine their impact on the properties of VGCCs. Data presented here show that phosphorylation at Thr498 in β_{2a} negatively regulates CaMKII binding both in *vitro* and *in situ* with a possible small effect of phosphorylation at other sites. Presumably, the conserved LXRXXS/T motif in β_{1b} is responsible for the regulated binding of CaMKII to this variant. Thus, interactions of CaMKII with β_2 and/or β_1 variants are regulated by phosphorylation, likely playing an important role in modulating CaMKII targeting to LTCCs and/or other VGCCs.

Interestingly, β subunits are not generally thought to be important in the regulation of T-type VGCCs. However, recent studies show that CaMKII directly interacts with and phosphorylates the II−III linker of the $Ca_V3.2$ α_1 subunit, shifting the current−voltage activation curve [75, 76]. In addition, recent data suggest that CaMKII may also interact with multiple cytoplasmic domains in $Ca_V1.2\alpha_1$ [147]. Thus, association of CaMKII with VGCC α_1 subunits may be an additional important feature allowing localized Ca^{2+} concentrations to feedback and regulate Ca^{2+} influx.

Phosphorylation of multiple sites in the $Ca_V1.2$ α_1 subunit by CaMKII appears to play a distinct role in voltage- and calcium-dependent modulation in heterologous cells [61, 63]. In addition, interactions of CaMKII with multiple intracellular domains of the $Ca_V1.2$ α_1 subunit have been implicated in LTCC facilitation[147]. In contrast, phosphorylation at Thr498 in β_{2a} is required for CaMKII to increase channel open probability at the single channel level and to facilitate whole cell Ca^{2+} currents in adult cardiomyocytes[79]. The present observations that phosphorylation at Thr498 appears to be relatively independent of the stable binding of CaMKII to β_{2a} (**Figures 12D and 13A**), and also inhibits CaMKII binding (**Figures 11, 12 and 13**), provide insights that may reconcile these seemingly disparate observations. Interestingly, phosphorylation of β_{2a} in preformed complexes promoted the slow dissociation of CaMKII (**Figure 14**). CaMKII dissociation might be required to allow protein phosphatases to act on the phospho-Thr498 site to reset channels to their basal state. Presumably, such a mechanism would be most relevant if Thr498 phosphorylation directly modulates LTCC properties, as suggested by the importance of Thr498 in CaMKII-mediated increases in LTCC open probability and LTCC current facilitation in adult cardiomyocytes[79]. Second, CaMKII may serve a structural role to dynamically assemble complexes containing additional, as yet unidentified, proteins associated with LTCCs. Thus, Thr498 phosphorylation in β_{2a} may promote reorganization of these protein complexes to enable facilitation. Structural roles for CaMKII have been postulated in neuronal postsynaptic densities[151]. Previous studies showed that disruption of the actin- or tubulin-

based cytoskeleton essentially abrogated CaMKII-dependent facilitation of cardiac LTCCs without affecting PKA-dependent facilitation, suggesting an important role for higher orders of molecular organization close to LTCCs in mediating the effects of CaMKII[144]. A final possibility is that dissociation from β_{2a} induced by Thr498 phosphorylation is important in allowing activated CaMKII to phosphorylate nearby regulatory sites in the α_1 or β subunits and/or other associated proteins. In other words, stable binding of activated CaMKII to β_{2a} may limit access of phosphorylation sites in other proteins to the CaMKII active site. Consistent with this hypothesis, dissociation of CaMKII from β_{2a} is substantially reduced by T498A mutation, perhaps explaining why CaMKII phosphorylates this mutant to a lower final stoichiometry than T498E-β_2 and L493A-β_2, which have much weaker interactions with CaMKII (**Figures 12 and 13**). Further studies will be needed to clarify the mechanism of CaMKII actions at VGCC complexes and, perhaps, to identify new CaMKII targets.

In summary, findings reported here and in other recent work (**see Introduction**) suggest that feedback regulation of Ca^{2+} influx via VGCCs is precisely controlled in specific subcellular microdomains by multiple mechanisms that allow CaMKII and other Ca^{2+}-dependent signaling proteins to associate with channel subunits. The precise nature of the feedback regulation by CaMKII seems likely to depend on the identity of the β subunit associated with the complex. The regulated interaction of activated CaMKII with β_1 and β_2 variants seems likely to be important, although phosphorylation of β_3 and β_4 may also play a role in some

cases. Our findings are in line with recent studies, suggesting that subcellular targeting of CaMKII via its interactions with CaMKAPs modulates the specificity of its downstream actions[159, 160]. These complex biochemical mechanisms for feedback regulation of Ca^{2+} influx via VGCCs presumably provide great flexibility for modulating a variety of downstream signaling events such as cardiac excitation−contraction and excitation−transcription coupling and neuronal synaptic plasticity. Moreover, alterations in the association of β subunits with VGCCs might disrupt feedback regulation and downstream signaling in heart failure and other diseases [161].

CHAPTER III

CAMKII ASSOCIATES WITH L-TYPE CALCIUM CHANNELS VIA SELECTED β SUBUNITS TO ENHANCE REGULATORY PHOSPHORYLATION

3.1 CHAPTER SUMMARY

Calcium/calmodulin-dependent kinase II (CaMKII) facilitates L-type Calcium Channel (LTCC) activity during normal cellular responses to stimuli but may also exacerbate LTCC-dependent pathophysiology. Facilitation of LTCCs by CaMKII requires the β_{2a} subunit of the channel and CaMKII binds to β_{1b} or β_{2a} subunits of LTCCs, but not the β_3 or β_4 subunits (**Chapter 2** and [150]) In this chapter I began to address the molecular mechanism of the β subunit-dependent LTCC facilitation by determining if the interactions of CaMKII and β_{1b} or β_{2a} localize CaMKII to the α_1 subunit of LTCC. I show that CaMKII reliably coimmunoprecipitates with LTCCs containing $Ca_V1.2$ α_1 and β_1 or β_2 subunits, but not β_4 from forebrain extracts. In addition, CaMKII can be tethered to the I/II linker of $Ca_V1.2\alpha_1$ and $Ca_V1.3\alpha_1$ subunits *in vitro* by β_1 and β_{2a} subunits but not by β_3 subunits. Moreover, CaMKII binding to the β_{2a} subunit is required for efficient targeting of CaMKIIα to the full-length $Ca_V1.2$ α_1 subunit in transfected HEK293 cells. The β_{2a}-dependent anchoring of CaMKII to LTCCs *in vitro* is regulated by phosphorylation of β_{2a} at Thr498, a site critical for CaMKII-dependent regulation of LTCCs. Even though CaMKII binds to the β_{2a} subunit of LTCC and phosphorylation of β_{2a} at Thr498 is required for LTCC facilitation (**see Introduction and Chapter 2**), the relationship between LTCC binding and

Thr498 phosphorylation remains unknown. In this chapter, I show that mutation of the β_{2a} subunit to disrupt CaMKII binding impairs the regulatory phosphorylation of β_{2a} at Thr498 without altering overall phosphorylation of $Ca_v1.2\alpha_1$ and β subunits. These findings provide a biochemical explanation for LTCC facilitation by CaMKII.

3.2 MATERIALS AND METHODS

Generation of plasmid constructs

A pLenti6-V5-D-Topo (Invitrogen, Carlsbad, CA) construct containing the rabbit cardiac $Ca_V1.2$ α_1 open reading frame (Accession Number X15539) with a surface HA-epitope between amino acids F709 and D710 of domain II S5-H5 extracellular loop (hereafter called HA-$Ca_v1.2\alpha_1$) was a generous gift of Dr. William Thiel[162]. The pCDNA6 construct encoding $Ca_v1.3$ was kindly provided by Dr. Diane Lipscombe. The I/II linker was amplified from the parent construct and inserted into a pGEX 4T-1 vector (Amersham Pharmacia Biotech, Uppsala, Sweden). The pGEX 4T-1 encoding the $Ca_v1.2$ AID was a generous gift of Dr. Rong Zhang. The β subunits were amplified by PCR and ligated into, pRSET A (Invitrogen), pHA-CMV5 (Clonetech), pFLAG-CMV2 (Sigma-Aldrich, St. Louis, MI) and pLenti6-V5-D-Topo. The pEGFP (Clonetech, Mount View, CA) construct encoding murine CaMKII with an N-terminal EGFP tag was made by Dr. Stefan Strack. The mCherry-tagged murine CaMKII constructs was made by Christopher Arnette. The pcDNA3 plasmid encoding a constitutively active T287D mutation of MYC-CaMKIIδ_2 was a gift from Dr. E. Olson. Dr. Gerald Zamponi provided the rat EGFP-$Ca_v1.2\alpha1$ construct. Site-directed mutagenesis were performed essentially as described in the QuikChange kit (Stratagene, La Jolla, CA).

Preparation of forebrain samples

The following procedure for preparation of Triton X-100 soluble fractions from rat or mouse brains was modified from Davare et al [163]. All the procedures were performed at 4^{o}C. One to two forebrains from seven to eight week old Sprague-Dawley rats (Pel-Freeze Biologicals, Rogers, Arkansas) or from 12 to 14-week old male C57B6J mice (Jackson Labs, West Grove, Pennsylvania) were pulverized under liquid nitrogen. The frozen powder was homogenized in Teflon-glass Potter-Elvehjem tissue grinders (Kontes, Vineland, NJ) in 5 to 10 ml of Solubilization Buffer (10 mM Tris-HCl, pH 7.5, 1% Triton X-100 (v/v), 20 mM EDTA, 10 mM EGTA, 100 µM PMSF, 500 µM benzamidine, 0.25 µM microcystin LR, 20 mM sodium β-glycerophosphate, 50 mM sodium fluoride, and protease inhibitor cocktail (Sigma, Catalog # P2714)). The homogenates were sonicated for one minute and centrifuged at 3600 x g (4000 rpm) for five minutes in a Beckman J-6B centrifuge using a JS-4.2 rotor to sediment cell/tissue debris and nuclei. Supernatants were removed and then centrifuged for 30 minutes at 250,000 x g in a Beckman L80K ultracentrifuge using a Ti70.1 rotor. The Triton-soluble supernatant was used as the input for immunoprecipitations, and the Triton-insoluble fraction was resuspended in solubilization buffer supplemented with 1% deoxycholate and centrifuged for 30 minutes at 250,000 x g. To prepare membrane enriched fractions for **Figure 15C**, the pulverized brains were resuspended in Membrane Buffer (300 mM sucrose, 75 mM NaCl, 20 mM EDTA, 10 mM EGTA, 100 µM PMSF, 500 µM benzamidine, 0.25 µM microcystin LR, 20 mM sodium β-glycerophosphate, 50 mM sodium fluoride, and protease inhibitor cocktail). Extracts were made and centrifuged as described above: supernatants were considered to be the cytosolic fraction, and the membrane enriched pellets were resolubilized in the Solubilization Buffer to make the membrane-enriched fraction.

Immunoprecipitations from forebrain

1 to 1.5 ml of Triton-soluble fraction of rat or mouse forebrains was precleared for 1 hour with protein-G Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and then incubated with 10 µg of either mouse (Jackson), rabbit or goat[164] control antibodies, rabbit anti-Cav1.2α_1 (AbCaM , Cambridge, MA), mouse monoclonal LTCC β subunit antibodies (Neuromab, Davis Ca), or an affinity purified goat antibody that recognizes all CaMKII isoforms[152]. The control, rabbit anti-$Ca_v1.2\alpha_1$ and the goat anti-CaMKII antibodies consisted of a mixture of IgG isotypes but the β_1 and β_2 antibodies consisted of IgG_{2a} isotype. After 1 hour, 10 µl of Protein-G agarose was added and the incubation continued for ~2 hours (or overnight for IPs from rat brain) at 4^{o}C. The resin was rinsed three times in 1 ml of solubilization buffer and bound proteins were analyzed by SDS-PAGE and western blotting with mouse antibodies to CaMKIIα (Affinity Bioreagents, Golden, Colorado) or LTCC subunits (α_1, β_1, and β_4: NeuroMab). A rabbit polyclonal β_2 antibody (a gift from Dr. Marlene Hosey) was used for the β_2 blot in **Figure 16A and 16B.**

Western Blotting

All samples were first separated in Tris-Glycine SDS-PAGE gels and then transferred to nitrocellulose membranes as described in **Chapter 2**. Membranes were blocked in 5% (w/v) milk in TTBS (50 mM Tris-HCl, pH 7.5; 150 mM NaCl, 0.1% (v/v) Triton-X-100), and then incubated with primary antibodies for 2 hours or overnight at 4ºC. After washing 3 times for ~5 minutes each, membranes were incubated for 30 to 60 minutes at room temperature with alkaline-phosphatase or horseradish peroxidase-conjugated secondary antibody. Membranes were washed at least four times each for ~10 minutes and developed colorimetrically

using BCIP/NBT or by enhanced chemiluminescence (Perkin-Elmer, Shelton, CT) respectively.

GST and His_6-tagged fusion protein expression and purification

Glutathione *S*-transferase (GST) and hexahistidine (His)-tagged fusion proteins were expressed in BL21-DE3 Gold *Escherichia coli* bacteria and purified using glutathione-agarose (Sigma, St Louis, MI) or His-Select Nickel Affinity Gel (Sigma), according to the manufacturers' protocol. Protein concentrations were determined by Bradford Assay (BioRad, Hercules, CA) or BCA assay (Pierce, Rockford, IL) using bovine serum albumin as a standard and validated by resolving proteins in SDS-PAGE gels and Coomassie-Blue staining.

CaMKII purification and autophosphorylation

Recombinant mouse CaMKIIα purified from baculovirus-infected Sf9 insect cells was autophosphorylated at Thr286 using ATP or [γ-^{32}P]ATP, essentially as described previously[152]. Briefly, CaMKII (1 μM subunit) was incubated on ice with 50 mM Tris, pH 7.5, 10 mM magnesium acetate, 1.5 mM $CaCl_2$, 20 μM calmodulin, 40 μM ATP, 1 mM DTT for 1.5 minutes and the reaction was stopped using 15 mM EDTA. For ^{32}P Thr286 autophosphorylation, CaMKII (5 μM subunit) was incubated on ice with 50 mM Tris, pH 7.5, 2 mM magnesium acetate, 1.5 mM $CaCl_2$, 20 μM calmodulin, [γ-^{32}P]ATP (20 μM, 40,000 cmp/pmol final) and 2 mM DTT. 5 μL of 100 mM ATP was added after 1 minute, and the reaction was continued for an additional minute before it was stopped with 10 mM EDTA. The radioactive CaMKII was desalted on a Sephadex G-50 column and used in the plate binding assay.

Glutathione-agarose cosedimentation assay

Thr286-autophosphorylated CaMKIIα was incubated with GST- I/II linker (or GST-AID) with or without His_6-tagged β_{2a} for one hour at 4^{o}C in GST Pull-down Binding Buffer(50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Triton X-100; 0.4 mL total volume). Glutathione-agarose (10 µL packed resin) was added, and the incubation was continued for one hour. Resin was collected by gentle centrifugation (1 min at 1000 rpm) and washed three times in binding buffer. Bound proteins were resolved by SDS-PAGE gel electrophoresis, transferred to nitrocellulose membranes and detected by Ponceau-S staining. Membranes were scanned and then the levels of all proteins were quantified by densitometry using ImageJ (NIH).

CaMKII plate binding assays

GST or GST-I/II proteins (100 pmol) were diluted into 0.2 ml of plate-assay buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, 0.1% (v/v) Tween-20, 5 mg/mL bovine serum albumin) and immobilized in glutathione-coated wells for 18-24 hours at 4^{o}C. [^{32}P-T^{286}]CaMKII (100 pmol) was premixed with buffer (no His-β_{2a}) or 36-100 pmol of His-β proteins. The mixture was added to the rinsed glutathione-coated wells. After a 2-hour incubation, unbound kinase and His-β were rinsed and bound [^{32}P-T^{286}] CaMKII was quantified by scintillation counting. In some cases, contents of the wells were eluted into SDS-PAGE loading buffer and analyzed by electrophoresis, western blotting and autoradiography.

Immunoprecipitations from HEK293 cells

HEK293 cells were cotransfected with DNA encoding HA-$Ca_v1.2\alpha_1$, FLAG-β_{2a} (WT, T498A, T498E or L493A) and EGFP-CaMKIIα or MYC-CaMKIIδ_2 (T287D), as indicated. In some cases CaMKII activation was enhanced by treating the cells for 5-minutes with 10 μM A23187. HA-$Ca_v1.2\alpha_1$ was immunoprecipitated from Triton-soluble cell extracts using 10 μg of anti-HA antibodies (Vanderbilt Monoclonal Antibody Core) or with 20 μl of anti-HA coated beads (Roche, Indianapolis, IN). Immunocomplexes were analyzed by western blotting for HA, FLAG, EGFP or MYC.

LTCC subunit phosphorylation in cells

In order to assess total phosphorylation of LTCC subunits, HEK293 cells (≈1 X 10^6 cells per well in a 6 well plate) were transfected with cDNAs for CaMKIIα (untagged or EGFP tagged, WT or T287D) or MYC-CaMKIIδ isoforms (WT or T287D) and β_{2a} (WT or L493A), with or without HA-$Ca_v1.2\alpha_1$. After 48 hours, intracellular phosphate pools were depleted by rinsing the cells once in phosphate-free DMEM (GIBCO) and then incubating in the same medium for 1 hour. The cells were then radioactively labeled with 0.42 mCi per well of ^{32}P-orthophosphate (PerkinElmer, Boston, MA) diluted in phosphate-free DMEM. After incubating for 4 hours at 37°C, radioactive medium was removed and the cells were rinsed twice in ice-cold PBS. The HA-$Ca_v1.2\alpha_1$ or FLAG subunit was immunoprecipitated from cell lysates and analyzed by SDS-PAGE. TOTAL ^{32}P-phosphorylation of LTCC subunits was detected by autoradiography, and total protein levels of each subunit were determined by western blotting with antibodies to the HA and FLAG epitopes. ImageJ was used to quantify β_{2a} ^{32}P-phosphorylation and protein levels, which were each corrected for background

determined by similarly quantifying signals in control immune complexes isolated from cells lacking β_{2a}. Total ^{32}P-phosphorylation of β_{2a} was then normalized to total β_{2a} protein levels in the LTCC immune complex.

For specific assessment of Thr498 phosphorylation in FLAG-β_{2a}, HA-$Ca_v1.2\alpha_1$ immune complexes were western blotted with anti-phospho-Thr286 CaMKII (Promega, Madison, WI) which we previously showed also recognizes the phosphorylated Thr498 residue in β_{2a} [150]. Immunoblots were quantitatively analyzed using ImageJ. Signal intensities for phospho-Thr498 and FLAG-β_{2a} were each corrected for backgrounds (estimated from control complexes isolated from cells lacking β_{2a}), and phospho-Thr498 signals were then normalized to β_{2a} signals in each LTCC immune complex.

Phosphorylation of β_{2a} with CaMKII

His-β_{2a} (WT or T498A) was incubated at 37^{o}C for 20 minutes with CaMKII in phosphorylation buffer (50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mg/ml bovine serum albumin, 0.5 mM $CaCl_2$, 1 μM CaM, 1 mM dithiothreitol, 0.4 mM [γ^{32}P]ATP (≈ 1000 cpm/pmol) or 0.4 mM ATP. The reaction was stopped by adding excess EDTA. Thr498 phosphorylation was verified using a Phospho-Thr286 CaMKII antibody that also detects phospho-Thr498 in β_{2a} [150].

CaMKII dissociation

In order to monitor dissociation of CaMKII-LTCC complexes, GST-$Ca_v1.2\alpha_1$I/II (WT or W470A: ≈10 pmol) was immobilized in glutathione-coated wells (Pierce, Rockford, IL). The wells were incubated for two hours at 4°C with [^{32}P-

T^{286}]CaMKII (0.25 μM) and His_6-β_{2a}(WT or T498A) in binding buffer (50 mM Tris-HCl pH 7.5 containing 150 mM NaCl, 0.25 mg/ml bovine serum albumin, 0.1% Triton X-100, 1 mM dithiothreitol, and 1 mM EDTA). The wells were then rinsed 8 times in binding buffer and CaMKII dissociation was initiated by addition of dissociation buffer (50 mM Tris-HCl pH 7.5, 0.1 M NaCl, 0.25 mg/ml bovine serum albumin, 0.1% Triton X-100, 1 mM dithiothreitol, 10 mM magnesium acetate) with or without 0.5 mM ATP. Soluble dissociated CaMKII was removed from the wells at the indicated times and quantified by scintillation counting. CaMKII that remained bound in the wells was also quantified similarly.

Statistics

Statistical analyses were performed with t-tests or analyses of variance (ANOVA) and post-hoc tests using Prism (Graphpad). P values less than 0.05 were considered significant.

3.3 RESULTS

Validation of antibodies against $Ca_v1.2\alpha_1$ and β subunits.

I tested the specificity of commercially available mouse monoclonal antibodies to the $Ca_v1.2\alpha_1$ by immunobloting heterologously-expressed HA-Cav1.2α_1. A blot with an antibody against the HA-tag detected expression of $Ca_v1.2\alpha_1$ in HEK293 cells (**Figure 15A**). The expressed HA-$Ca_v1.2\alpha_1$ was efficiently detected by the antibody against $Ca_v1.2\alpha_1$. I also tested the specificity of commercially available mouse monoclonal antibodies raised against β_1, β_2 and β_4 subunits by immunobloting bacterially-expressed GST fusion proteins containing the full length amino acid sequences of all four β subunit isoforms (**Figure 15B**). The specificity of the β_1 and β_4 antibodies was also verified using subcellular fractions of mouse brain and extracts of HEK293 cells transfected to express recombinant β_1 or β_4 (**Figure 15C**). Each antibody specifically detected the respective isoform, albeit with different sensitivity, either as GST fusion proteins or recombinant proteins expressed in HEK293 cells. Moreover, the β_1 and β_4 antibodies recognized a closely migrating series of bands that were enriched in brain particulate/membrane fractions with appropriate apparent molecular weights to represent a mixture of splice variants and/or post-translationally modified forms of the respective β subunit. The mouse anti-β_2 antibody was omitted from the further verification because it only weakly detected β_{2a} (**Figure 15B**). Instead, the limited amount of rabbit β_{2a} antibody (from Dr. Marlene Hosey) was used because it strongly detected purified GST-β_{2a}, FLAG-β_{2a} expressed in HEK

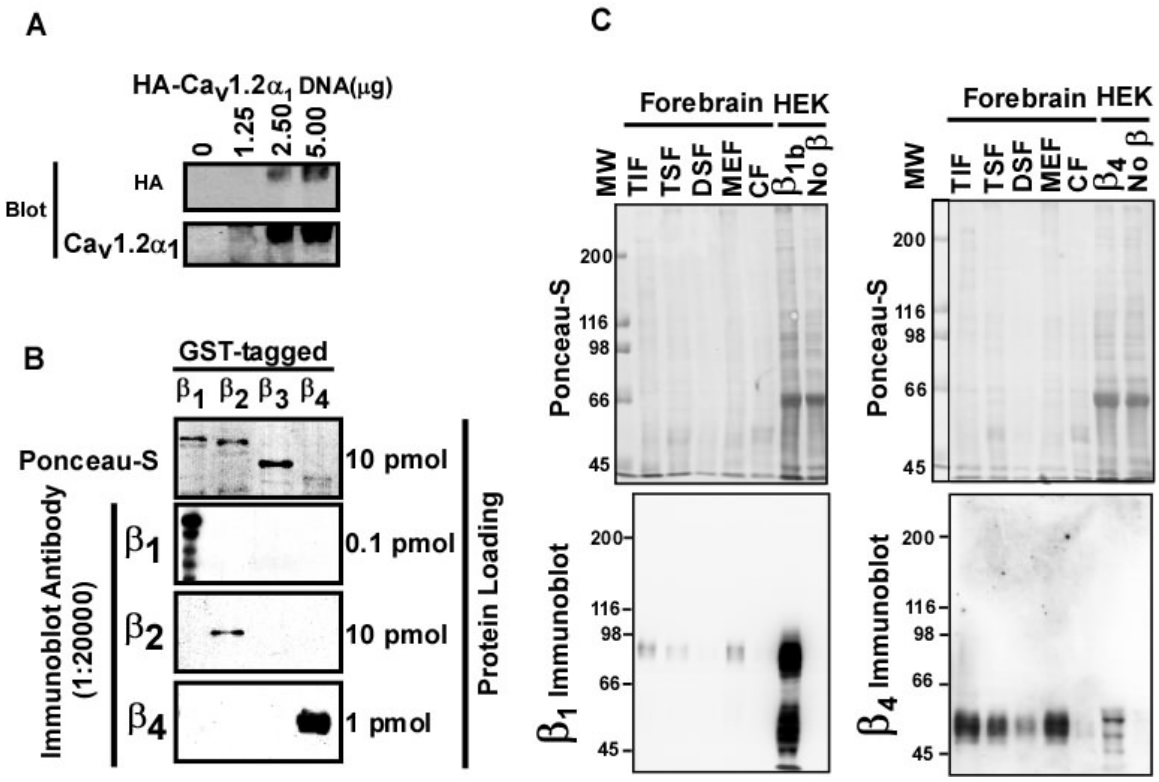

Figure 15. Specificity of α_1 and β subunit antibodies.
(A) Testing of $Ca_v1.2\alpha_1$ subunit antibody. HEK293 cells transfected with 0, 1.25, 2.5 or 5 μg of HA-$Ca_v1.2\alpha_1$ DNA were blotted with antibodies against HA (1:2000) or the $Ca_v1.2\alpha_1$ from NeuroMab (1:2000).

(B) Purified GST-β_{1b}, β_{2a}, β_3 and β_4 (0.1-10 pmol, as indicated) were immunoblotted with NeuroMab antibodies to the β_1, β_2, or β_4 subunit isoforms. The top image shows Ponceau-S staining of GST-β proteins.

(C) Subcellular fractions of mouse forebrain and lysates of HEK cells expressing Flag-β_1, HA-β_4 or no β were western blotted using the β_1 (left) or β_4 (right) subunit antibodies (1:1000): Triton-insoluble fraction (TIF), Triton-soluble fraction (TSF), deoxycholate-soluble fraction (DSF), membrane enriched fraction (MEF) or cytosolic fraction (CF). Top panels show Ponceau-S stained membranes of total protein loaded.

cells and a band at the expected molecular weight of native cardiac or brain β_2 (*data not shown*).

Association of CaMKII with forebrain LTCC complexes containing β_1 or β_2 subunits

LTCC α_1 subunits and a heterogenous mixture of β subunits are expressed in the brain. In order to determine whether CaMKII associates with LTCCs in forebrain, $Ca_v1.2\alpha_1$ immunoprecipitates from rat forebrain Triton-soluble extracts were probed with anti-phospho-Thr^{286} CaMKII or rabbit anti-β_2 antibodies. Bands that corresponded to phospho Thr^{286}-CaMKII and phospho Thr^{498}-β_{2a} were detected in the complexes, along with total β_{2a} (**Figure 16A**). (The same phospho-Thr antibody detects both CaMKII and β_{2a} phosphorylation, and is described in more detail later in this chapter). These preliminary findings were validated using a reverse coimmunoprecipitation where CaMKII immune complexes isolated from forebrain extracts were immunoblotted for LTCC subunits. CaMKII was substantially depleted from the supernatant, with a corresponding enrichment in the immune complexes. Control IgGs failed to deplete CaMKII from the supernatant: the weak detection of CaMKII in control IgG complexes, despite extensive washing, likely reflects the abundance of CaMKIIα in forebrain. Thus, CaMKII was substantially and specifically enriched in CaMKII complexes. Notably, the $Ca_V1.2\alpha_1$ subunit was readily detected in CaMKII complexes, but not in control samples (**Figure 16B and C**). Initial experiments using limited available quantities of a rabbit antibody to the β_2 subunit (see Experimental Procedures) and a mouse β_1 subunit antibody demonstrated that rat CaMKII complexes also contained β_1 and β_2 subunit variants (**Figure 16B**). CaMKIIα and LTCC subunits were present in the same fractions from mouse forebrain and were coimmunoprecipitated using CaMKII antibodies. (**Figure 16C and A3**). If

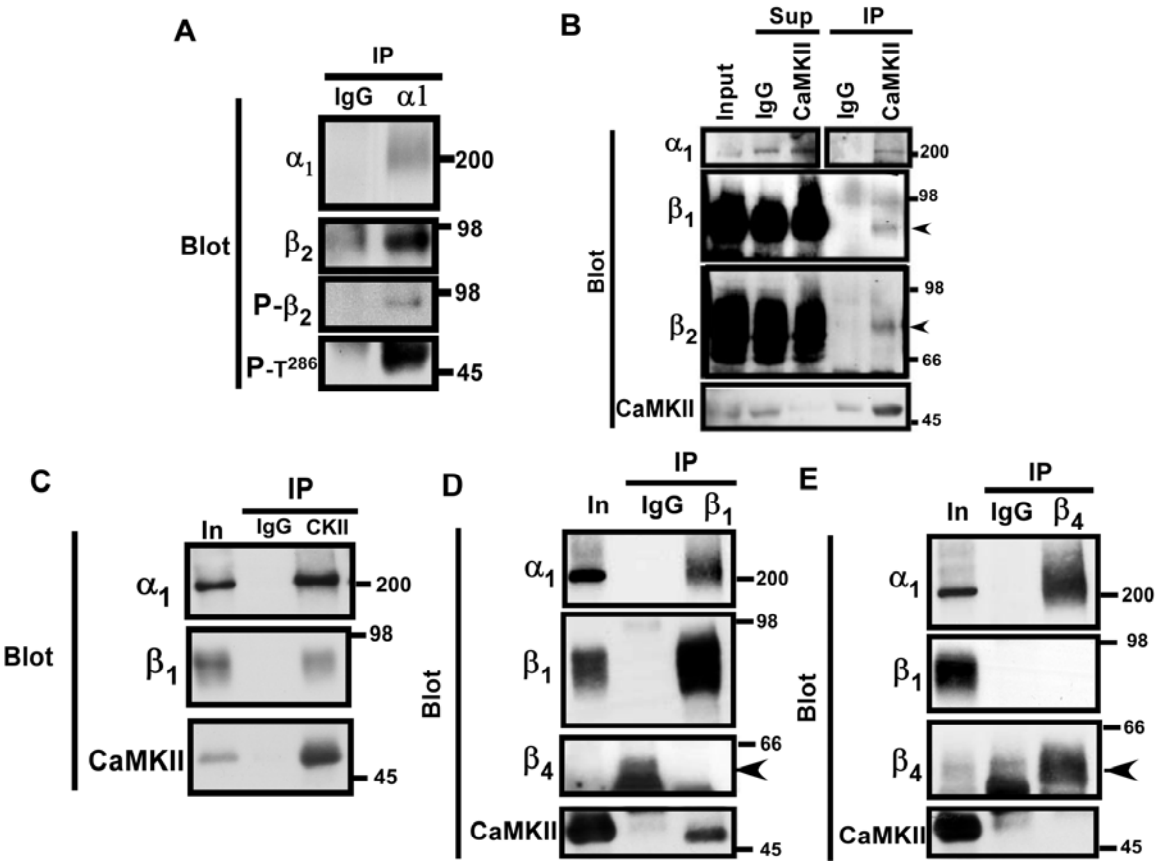

Figure 16. CaMKII association with LTCC subunits in brain.
Triton-soluble extracts of rat (**A, B**) or mouse (**C-E**) forebrains were immunoprecipitated using antibodies raised against $Ca_v1.2$ (**A**), CaMKII (CKII, **B,C**), β_1 (**D**) or β_4 (**E**). The immune complexes were western blotted for calcium channel subunits (1:1000) and anti-CaMKII (1:2000). These data are representative of 1 (**A**), 2 (**B**) or more than 4 (**C-E**) experiments.

LTCCα_1-β-CaMKII complexes exist in brain, it should be possible to isolate them not only using $Ca_v1.2\alpha_1$ and CaMKII but also using the β subunit. The mouse β_1 and β_4 subunit antibodies were then used to immunoprecipitate calcium channel complexes from a Triton-soluble fraction of mouse brain. Western blots demonstrated that the appropriate β subunit isoform was specifically enriched in each immune complex (**Figure 16D and E**). The $Ca_v1.2\alpha_1$ subunit was substantially enriched in both immune complexes, though somewhat more so in β_4 complexes than in β_1 complexes. In contrast, CaMKIIα was specifically detected in β_1 immune complexes (**Figure 16D**) but not in β_4 immune complexes (**Figure 16E**).

The β subunits differentially tether CaMKII to LTCC α_1 subunits

I then investigated the role of direct interactions between the $Ca_v1.2$ α_1 and β subunits and/or CaMKII in the assembly of the CaMKII-LTCC complex. I previously showed that Thr287-autophosphorylated CaMKIIδ directly interacts with the β_{1b} and β_{2a} subunits with similar affinities (K_d ≈100 nM CaMKII subunit), but not with β_3 or β_4 subunits (**Chapter 2 and** [150]). The GK domains in β subunits bind to I/II linkers of VGCC α_1 subunits with high but variable (K_d ≈ 5-60 nM) affinities [25, 60, 165, 166]. In addition, CaMKII was previously shown to interact directly with multiple intracellular domains of the $Ca_V1.2$ α_1 subunit *in vitro*, including the I/II linker, although affinities of these interactions were not determined[147]. As a first step toward dissecting roles of these interactions in assembly of CaMKII-LTCC complexes, we investigated the binding of Thr286 autophosphorylated CaMKIIα to the GST-tagged I-II linker domain of the $Ca_V1.2$ α_1 subunit (GST-I/II) in the absence or presence of purified hexahistidine (His)-

tagged β_{1b}, β_{2a} and β_3 subunits (we were unable to purify His-tagged β_4). There was no detectable direct interaction of CaMKIIα with GST-I/II, but addition of His-β_{1b} or His-β_{2a}, but not His-β_3, allowed for the immobilization of CaMKII by GST-I/II (**Figure 17A and C**). Even though the affinity of β_1 for CaMKII is nearly three-fold higher than of β_2, His-β_{1b} immobilized less CaMKII than His-β_2 under these conditions, possibly due to the weaker binding of His-β_1 to GST-I/II (**Figure 17B**). Notably, in this series of experiments His-β_3 bound to the I/II linker at least as well as His-β_{2a}, but was completely ineffective in immobilizing CaMKIIα on the I/II linker (**Figure 17B**). Additional GST-cosedimentation analyses confirmed that there was no significant difference in binding of His-β_{2a} and -β_3 subunits to the I/II linker (**Figure 17D**, His-β_3 binding was 85±15% of His-β_{2a} binding: mean±sem, n=3. p=0.43). Thus, CaMKII can be tethered to the I/II linker of $Ca_V1.2$ α_1 subunits by specific β subunit isoforms *in vitro.*

The β_{1b} and β_{2a} subunit differentially target CaMKII to recombinant full-length α_1

In **Figure 17** I observed that the β_{1b} and β_{2a} subunits differentially associated with the $Ca_v1.2\alpha_1$ I/II linker domain. Since the I/II linker is the primary interaction domain of $Ca_v1.2\alpha_1$ and β subunits, a deficit in binding to the I/II linker might translate into a deficit in binding to the full-length channel. I therefore compared association of FLAG-β_{1b} and FLAG-β_{2a} with HA- $Ca_v1.2\alpha_1$ in HEK cells. Much less FLAG-β_{1b} coimmunoprecipited with HA-$Ca_v1.2\alpha_1$ than did FLAG-β_{2a}.

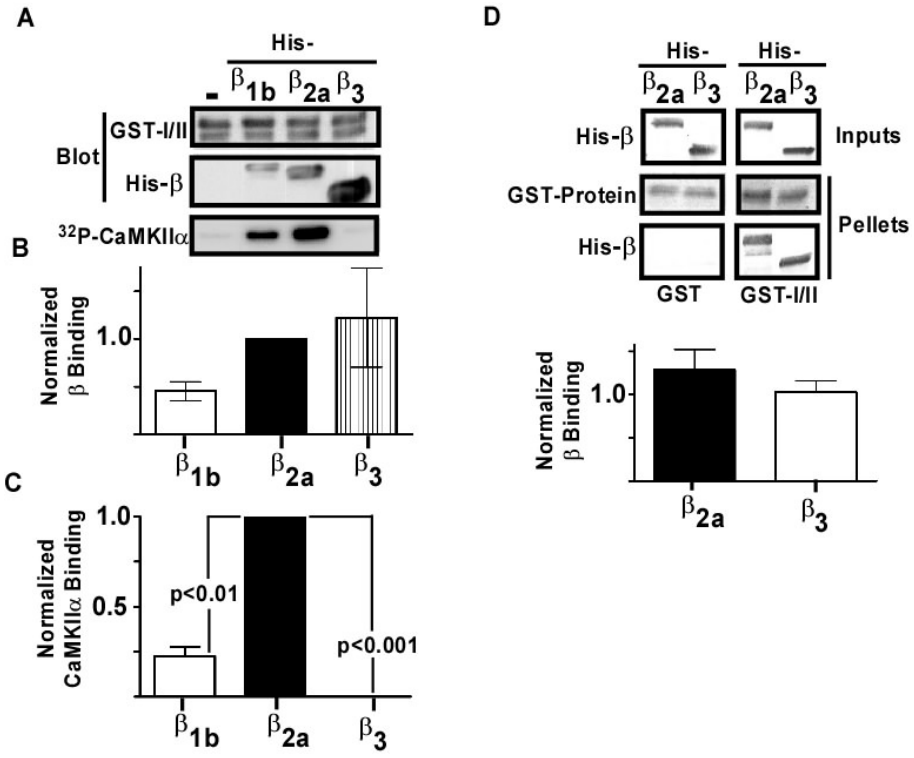

Figure 17. β subunits selectively anchor CaMKII to the α_1 I/II linker *in vitro*. GST-I/II (100 pmol) immobilized in glutathione-coated wells was incubated with a mixture of [^{32}P-T^{286}]CaMKIIα (100 pmol) and either His-β_1, His-β_2 or His-β_3 (36 pmol) in the presence of EDTA to prevent protein phosphorylation.

(**A**) Bound proteins were eluted with SDS and analyzed by western blotting with anti-GST or anti-His antibodies, and by autoradiography. (Top: blots are representative of 3 experiments. Bottom: autoradiograph of samples from one of the three experiments quantified in C)

(**B)** The amounts of bound His-β subunits were quantified and normalized to the amounts of immobilized GST-I/II and of His-β in the input: binding of β_{2a} was set to 1.0. A one-sample t-test was used to compare the binding of β subunits to a theoretical value of 1.0. (n = 3 for β_{1b} and β_{2a}, n = 2 for β_3 binding)

(**C**) Bound [^{32}P-T^{286}]CaMKIIα also was quantified by scintillation counting and normalized to the amount of bound His-β: CaMKII binding in the presence of β_{2a} was set to 1.0. The mean±sem of 3 experiments (2 in the case of β_3) is plotted in B/C. Binding of CaMKII was analyzed by ANOVA followed by Newman-Keuls multiple comparisons test.

(**D**) 100 pmol of GST or GST-I/II linker was incubated with His-β_{2a} or His-β_3 and binding of the β subunits to the I/II linker in a glutathione-agose pull down assay was quantified. (n =3)

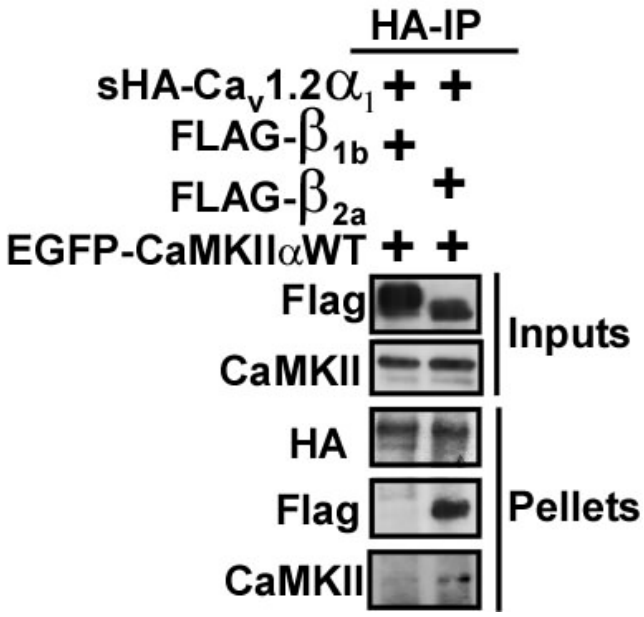

Figure 18. The β_{1b} and β_{2a} subunit differentially target CaMKII to full-length LTCCα_1 subunits in heterologous cells.
HEK293 cells we transfected with DNA encoding EGFP-CaMKIIα, HA-$Ca_v1.2$ α_1 and FLAG-β_{1b} or FLAG-β_{2a} as indicated. Triton-soluble fractions (Inputs) and anti-HA immune complexes (pellets) were western blotted for HA, FLAG, or EGFP. These data are representative of 4 similar experiments.

In parallel, much less CaMKII was associated with HA-$Ca_v1.2\alpha_1$ in the presence of FLAG-β_{1b} (**Figure 18**). Thus β_{1b} targets less CaMKII to $Ca_v1.2\alpha_1$ than β_{2a} does despite the higher apparent affinity of β_{1b} for autophosphorylated CaMKII *in vitro*(**Figure 10D**).

Mechanism of β_{2a}-dependent tethering of CaMKIIα to the I/II linker

Since the β_{2a} subunit most strongly tethers CaMKII to the $Ca_V1.2$ α_1, and is essential for CaMKII-dependent regulation of cardiac LTCCs, I used β_{2a} to explore the mechanism of β_{2a}-dependent tethering of CaMKIIα to $Ca_V1.2$ α_1 subunits. First, I examined the possibility that direct interactions of activated CaMKIIα with parts of the I/II linker domain other than the minimal 18-amino acid AID that is sufficient for binding to β subunits[25, 60] may be required for stable CaMKII tethering to the I/II linker. I performed experiments using a GST fusion protein containing the AID and failed to detect direct interactions of activated CaMKIIα with GST-AID but His-β_{2a} was able to tether activated CaMKII to GST-AID (**Figure 19A and C**). This implies the core AID of the α_1 subunit is sufficient for β subunit-dependent anchoring of CaMKII to the subunit.

Mutation of Trp470 to Ala in the I/II linker severely compromises the binding of β subunits[60, 165, 166], and the W470A mutation essentially abrogated the interactions of both His-β_{2a} and activated CaMKII with GST-I/II (**Figure 20A**). The high affinity direct binding of activated CaMKIIα to β_{2a} is disrupted by Leu493 to Ala mutation[150]: the L493A mutation significantly reduced β_{2a}-dependent cosedimentation of activated CaMKIIα with GST-AID by about 70% (**Figure 19A and C**) and with GST-I/II by about 60% (**Figure 20A and C**). Notably, disruption

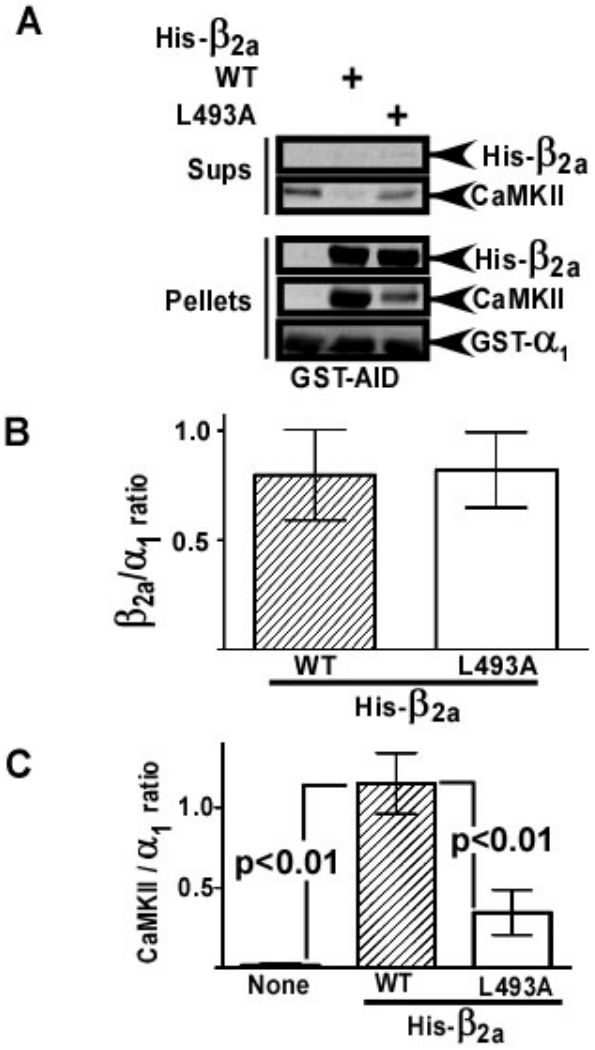

Figure 19. β_{2a} anchors CaMKII to the α_1 AID in vitro.
(**A**) Thr286-autophosphorylated CaMKIIα (100 pmol) was incubated with GST-AID with or without His-tagged β_{2a} (WT or L493A). Complexes isolated using glutathione agarose were western blotted as indicated.

(**B**) Quantitative comparison of β_{2a}(WT) and β_{2a}(L493A) binding to GST-AID.

(**C**) Quantitative comparison of CaMKII binding to GST-AID under different conditions. The mean±sem from 4 experiments is plotted in panels B/C. Data were compared by one-way ANOVA followed by Newman-Keuls multiple comparisons test.

of CaMKII binding with the L493A mutation had no detectable effect on the amount of β_{2a} that co-sedimented with GST-I/II (**Fig. 20B**) or with GST-AID (**Figure 19B**).

I then used a more quantitative glutathione-coated multi-well plate binding assay (see Methods) to further explore the mechanisms underlying His-β_{2a}-dependent tethering of activated ^{32}P-labeled CaMKII to GST-I/II. Consistent with cosedimentation assays, activated CaMKII efficiently bound to GST-I/II in the presence of His-β_{2a}, and the omission His-β_{2a} reduced binding by over 100-fold (**Figure 20D**).The His-β_{2a}-dependent binding of CaMKII to GST-I/II also was significantly compromised by L493A mutation of His-β_{2a} (≈ 50% reduction in binding). Moreover, a His-β_{2a}(410-505) protein which lacks the SH3 and GK domains and cannot bind to the AID (data not shown), but contains the CaMKII-binding domain[150], failed to support the tethering of activated CaMKII to GST-I/II (**Figure 20D**). The I/II linker of rabbit $Ca_v1.2\alpha_1$ is highly conserved with that of rat $Ca_v1.3\alpha_1$ but not identical (however, the 18-amino acid AID of Cav1.2 and $1.3\alpha_1$ are identical). The His-β_{2a} also tethered CaMKII to the GST-I/II linker of $Ca_v1.3\alpha_1$ (**Figure 20D**). Taken together, these findings show that β_{2a} subunit can serve as an adaptor protein to tether CaMKII to the I/II linker domain of the $Ca_v1.2$ and $Ca_v1.3$ α_1 subunits.

In the binary CaMKII-β_{2a} interactions in **chapter 2**, the Leu493A mutation reduced CaMKII binding by over 90% (**Figure 11C, 12C and 13C**) but the same mutation reduced CaMKII binding by only ~50-70% in the ternary complex. Thus β subunit binding to the I/II linker appeared to enhance the binding of CaMKII to the β subunit. I therefore tested if CaMKII binding may similarly enhance the binding of the β subunit for the I/II linker. CaMKIIα binding to His-β_{2a} did not

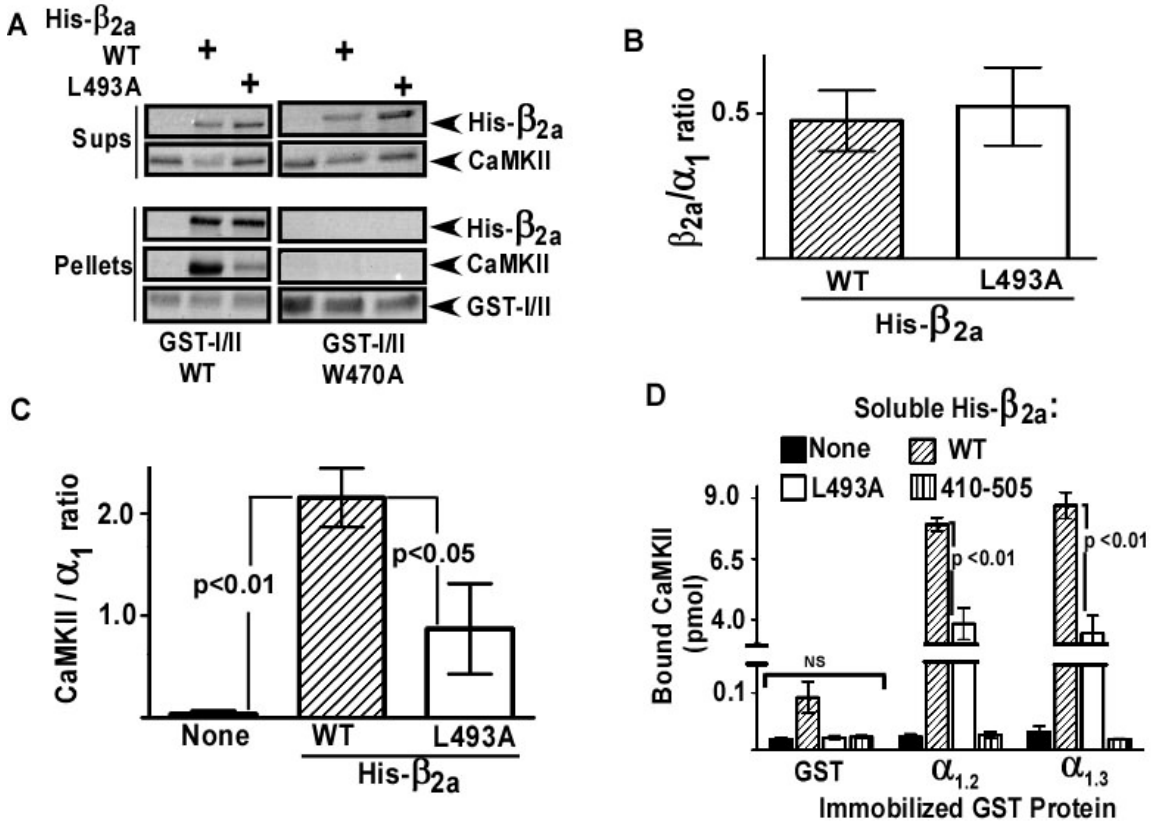

Figure 20. Molecular determinants of CaMKII anchoring to the α_1 I/II linker.
(**A**) Thr286-autophosphorylated CaMKIIα (100 pmol) was incubated with GST-I/II (WT or W470A) (100 pmol) with and without His-tagged β_{2a} (WT or L493A) (100 pmol). Complexes were isolated on glutathione agarose and the bound (pellets) and unbound (sups) proteins were detected by Ponceau-S staining.

(**B**) Quantification (mean±sem, n=3) of β_{2a}(WT) and β_{2a}(L493A) binding to GST-I/II.

(**C**) Quantification (mean±sem, n=3) of CaMKIIα binding to GST-I/II: data were analyzed by one-way ANOVA followed by Newman-Keuls multiple comparisons test.

(**D**) GST or GST-$Ca_v1.2\alpha_1$I/II (100 pmol) were immobilized in glutathione-coated wells and incubated with a mixture of [^{32}P-T^{286}]CaMKIIα (100 pmol) and either His-β_{2a} (WT or L493A) or His-β_{2a}(410-505) (100 pmol) in the presence of EDTA. Bound [^{32}P-T^{286}]CaMKII was quantified by scintillation counting. Data (mean±sem, n=3) were analyzed by two-way ANOVA with Bonferroni posttest.

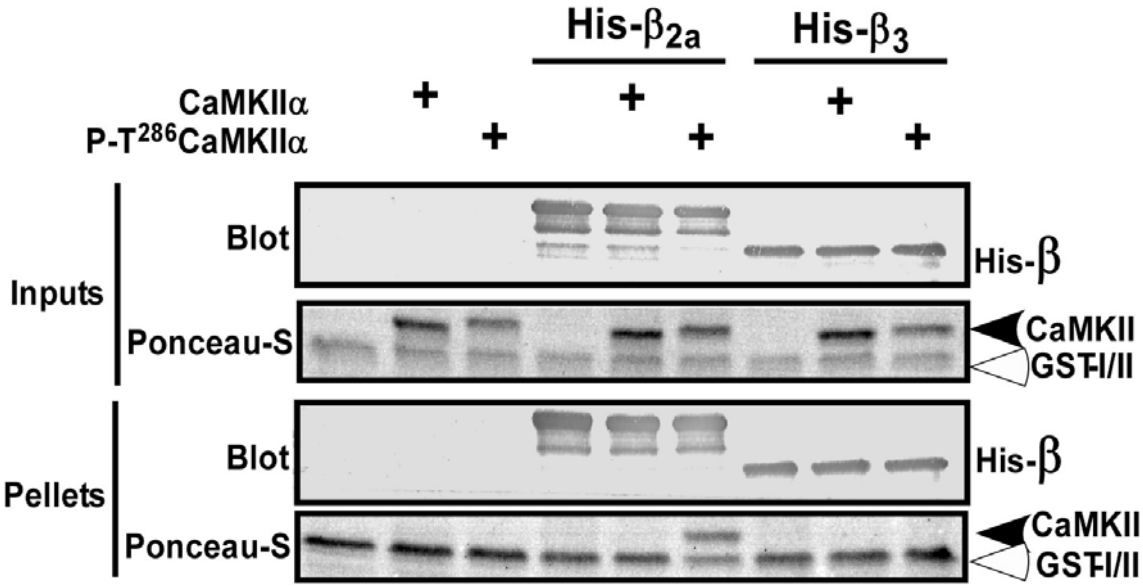

Figure 21. CaMKII binding does not affect β subunit binding to the α_1 I/II linker.
His-tagged β_{2a} or β_3 (100 pmol) was incubated with GST-I/II linker with nophosphorylated or Thr286-autophosphorylated CaMKIIα (100 pmol) was incubated with GST-I/II (100 pmol). Complexes were isolated on glutathione agarose and the bound (pellets) and total proteins were detected by Ponceau-S staining and western blotting. Blot representative of three similar experiments

affect the interaction of His-β_{2a} with the I/II linker (**Figure 21**). Notably, these data are also consistent with the equivalent association of His-β_{2a} WT and His-β_{2a} (L493A) with the I/II linker (**Figure 20**).

The β_{2a} subunit enhances CaMKII association with full-length $Ca_V1.2$ α_1

Next, I tested whether the β_{2a} subunit also functions as an adapter protein to tether CaMKII to full-length $Ca_v1.2\alpha_1$ subunits. Although previous co-immunoprecipitation studies showed that CaMKII associates with LTCC complexes in the presence of β_2 subunits[63, 147], the role of the β subunit in formation of this complex was not explored. Therefore, I transfected HEK293 cells to express EGFP-CaMKII in the absence or presence of HA-$Ca_v1.2\alpha_1$ subunits and WT or L493A mutated FLAG-β_{2a}. The cells were pretreated with A23187 Ca^{2+} ionophore to boost CaMKII activation because under basal conditions CaMKII was only weakly detected in LTCC complexes (**Figures 8, 18A and A4**). After the pretreatment, LTCC complexes were isolated by immunoprecipitation using anti-HA antibodies and immunoblotted for all three proteins. In some cases, HA-$Ca_v1.2\alpha_1$ subunits could not be detected in cell extracts due to the relatively low expression levels, but were readily detected in HA-immune complexes. As expected due to the known roles of β subunits in modulating surface expression and trafficking of VGCC α_1 subunits, co-expression of β_{2a} enhanced the levels of α_1 subunit expression. WT and L493A mutated FLAG-β_{2a} proteins were expressed at similar levels and induced a similar increase in α_1 subunit expression (**Figure 22, A4, and A5**). (Note: HA-immune complexes from HEK293 cells that were not transfected to express FLAG-β_{2a} contained a weak, non-specific FLAG-immunoreactive protein of about 70 kDa.) Total expression levels of CaMKII were similar in all transfections. However, CaMKIIα were only reliably detected in anti-HA immune complexes

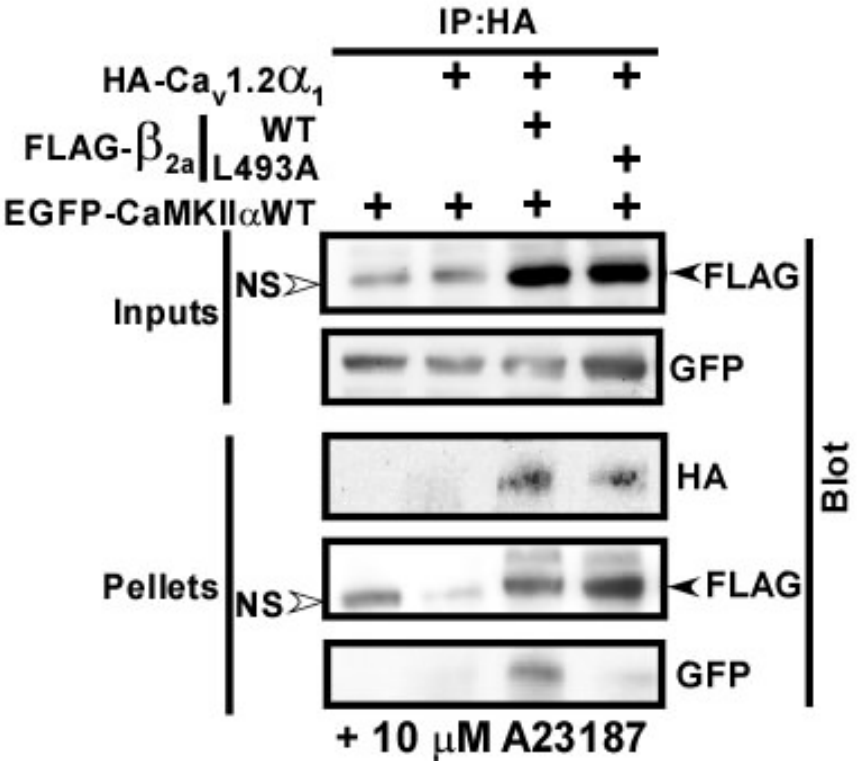

Figure 22. The β_{2a} subunit targets CaMKIIα to LTCCα_1 subunits in heterologous cells.
HEK293 cells co-expressing EGFP-CaMKIIα, HA-$Ca_v1.2$ α_1 and/or FLAG-β_{2a} (WT or L493A) as indicated were treated with A23187. Triton-soluble fractions (Inputs) and $Ca_v1.2\alpha_1$ immune complexes (pellets) were western blotted for HA, FLAG, or EGFP. These data are representative of at least 3 independent experiments.

isolated from cells expressing WT FLAG-β_{2a} (**Figure 22**). Much lower levels of CaMKIIα were detected in anti-HA immune complexes isolated from cells expressing L493A-mutated FLAG-β_{2a} or from cells that did not express FLAG-β_{2a} (**Figure 22**). Similar results were obtained using activated CaMKIIδ isoform (**Figure A5**). These data demonstrate that high affinity interaction with the β_{2a} subunit is necessary for its efficient assembly of CaMKII with the LTCC complex.

CaMKIIα binding to β_{2a} does not affect overall phosphorylation of $Ca_V1.2\alpha_1$ and β_{2a} subunit

LTCCs are regulated by several kinases that phosphorylate multiple sites in both the α_1 and β subunits[24]. More specifically, CaMKII-dependent regulation of LTCCs has been linked to phosphorylation of the $Ca_V1.2$ α_1 subunit at Ser1512 and Ser1570[63] and of the β_{2a} subunit at Thr498, although several additional sites of unknown function in β_{2a} can be phosphorylated by CaMKII[79]. In order to test the hypothesis that CaMKII binding to β_{2a} modulates LTCC subunit phosphorylation, HA-$Ca_V1.2$ α_1, EGFP-CaMKII and/or FLAG-β_{2a} (WT or L493A) were co-expressed in HEK293 cells. After labeling cells with ^{32}P-orthophosphate (see Methods), LTCC complexes were immunoprecipitated from cell extracts for analysis by SDS-PAGE followed by autoradiography to detect ^{32}P-phosphorylated proteins. The ^{32}P-labeling of $Ca_V1.2\alpha_1$ in the absence of β_{2a} and EGFP-CaMKII indicated that endogenous kinases significantly phosphorylate $Ca_V1.2\alpha_1$ subunits under basal incubation conditions **(Figure 23**). Co-expression of EGFP-CaMKII had no consistent effect on ^{32}P-phosphorylation of $Ca_V1.2\alpha_1$ in the absence or presence of β_{2a} (WT or L493A). Moreover, the ^{32}P-phosphorylation of β_{2a} within LTCC complexes was not significantly altered by expression of EGFP-CaMKII or by the L493A mutation (**Figure 24**). However, we confirmed that EGFP-CaMKII associated with LTCC complexes containing

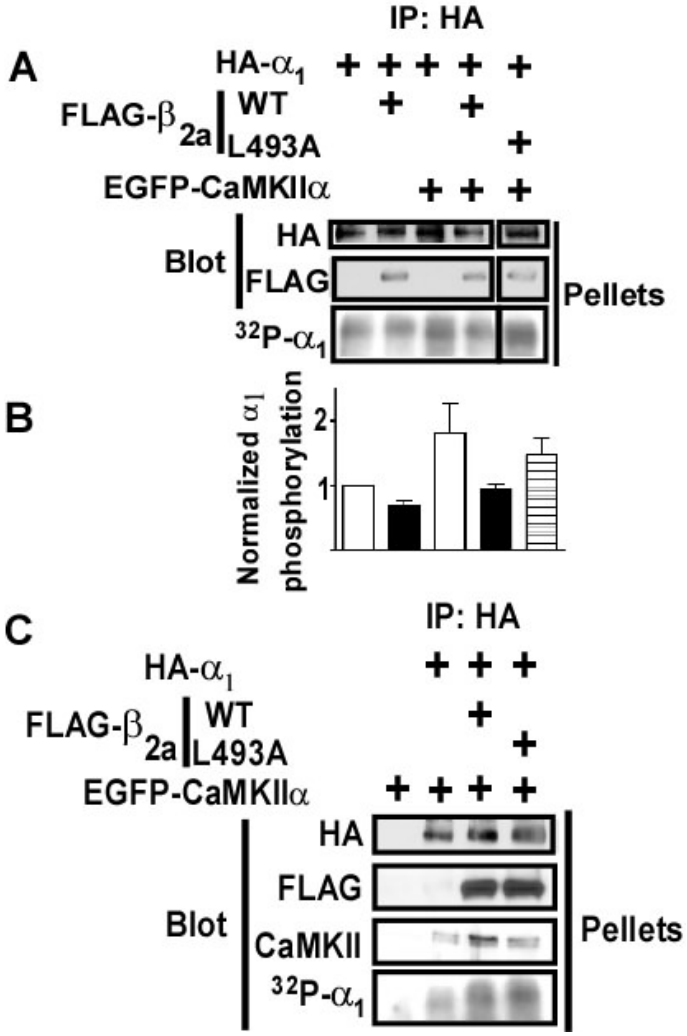

Figure 23. CaMKII binding to the β_{2a} subunit does not affect total phosphorylation of the α_1 subunits.

(**A**)HEK293 cells co-expressing HA-$Ca_v1.2$ α_1, EGFP-CaMKIIα and FLAG-β_{2a} (WT or L493A) were labeled with ^{32}P-phosphate (see Methods). Anti-HA immune complexes were analyzed by SDS-PAGE and autoradiography to detect phosphorylation of $Ca_v1.2$ α_1. Antibody dilutions: Anti-HA (1:2000), Anti-FLAG (1:5000)

(**B**)The ^{32}P labeling of $Ca_v1.2\alpha_1$ subunits in **A** was quantified from four independent experiments plotted. The mean+/- SEM is plotted.

(**C**) Anti-HA immune complexes from one of the experiments in **A** were probed for FLAG and CaMKII. Antibody dilutions: Anti-HA (1:2000), Anti-FLAG (1:5000), Anti-CaMKII (1:2000)

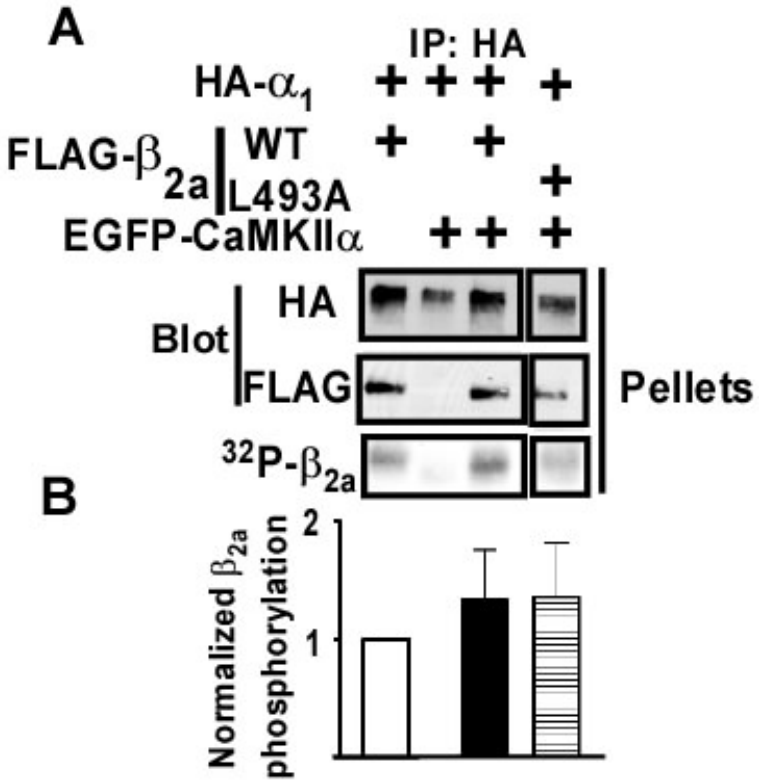

Figure 24. CaMKII binding to the β_{2a} subunit does not affect total phosphorylation of the β subunits.
(**A**) HEK293 cells co-expressing HA-$Ca_v1.2$ α_1, EGFP-CaMKIIα and FLAG-β_{2a} (WT or L493A) were labeled with ^{32}P-phosphate (see Methods). Anti-HA immune complexes were analyzed by SDS-PAGE and autoradiography to detect phosphorylation of β_{2a} subunits. Antibody dilutions: Anti-HA (1:2000), Anti-FLAG (1:5000), Anti-CaMKII (1:2000)

(**B**) Phosphate incorporation into β_{2a} in 4 independent experiments was quantified and normalized to wild-type β_{2a} phosphorylation in the absence of EGFP-CaMKIIα: the mean±sem is plotted.

WT β_{2a} subunits (**Figure 23C**). Thus, total ^{32}P-phosphorylation of $Ca_v1.2\alpha_1$ and β_{2a} subunits in LTCC complexes did not appear to be affected by CaMKII association via β_{2a} under basal conditions. CaMKIIα binding to β_{2a} selectively enhances phosphorylation of β_{2a} at Thr498.

Total ^{32}P-phosphorylation of proteins in ^{32}P-orthophosphate labeling experiments can report the phosphorylation of multiple sites in a target protein. However, β_{2a} can be phosphorylated at multiple serine and threonine residues by other kinases, such as PKA (at Ser 458,478,479) [78] and PKG (at Ser 496) [62]. Therefore, we reasoned that changes in phosphorylation at a limited number of sites due to L493A mutation may be obscured by ^{32}P-phosphorylation at other sites in β_{2a}. We previously showed that the critical regulatory phosphorylation of β_{2a} at Thr498 can be detected using a phospho-Thr286 CaMKIIα antibody (due to the similarity of surrounding amino acid sequences) and that L493A mutation does not affect recognition of phospho-Thr498 in β_{2a} [150].

Therefore, I used the anti-phospho-Thr286 CaMKIIα antibody to specifically examine Thr498 phosphorylation in β_{2a} within LTCC complexes immunoprecipitated from transfected HEK293 cells. I took a number of steps to verify that the anti-phospho-Thr286 CaMKIIα antibody specifically detected Thr498 phosphorylation but not the co-migrating EGFP-CaMKII. First, I verified that under our experimental conditions (expression levels, protein loading and chemiluminescence exposure levels) the antibody did not reveal Thr286 autophosphorylation of EGFP-CaMKII in inputs, even when CaMKII was

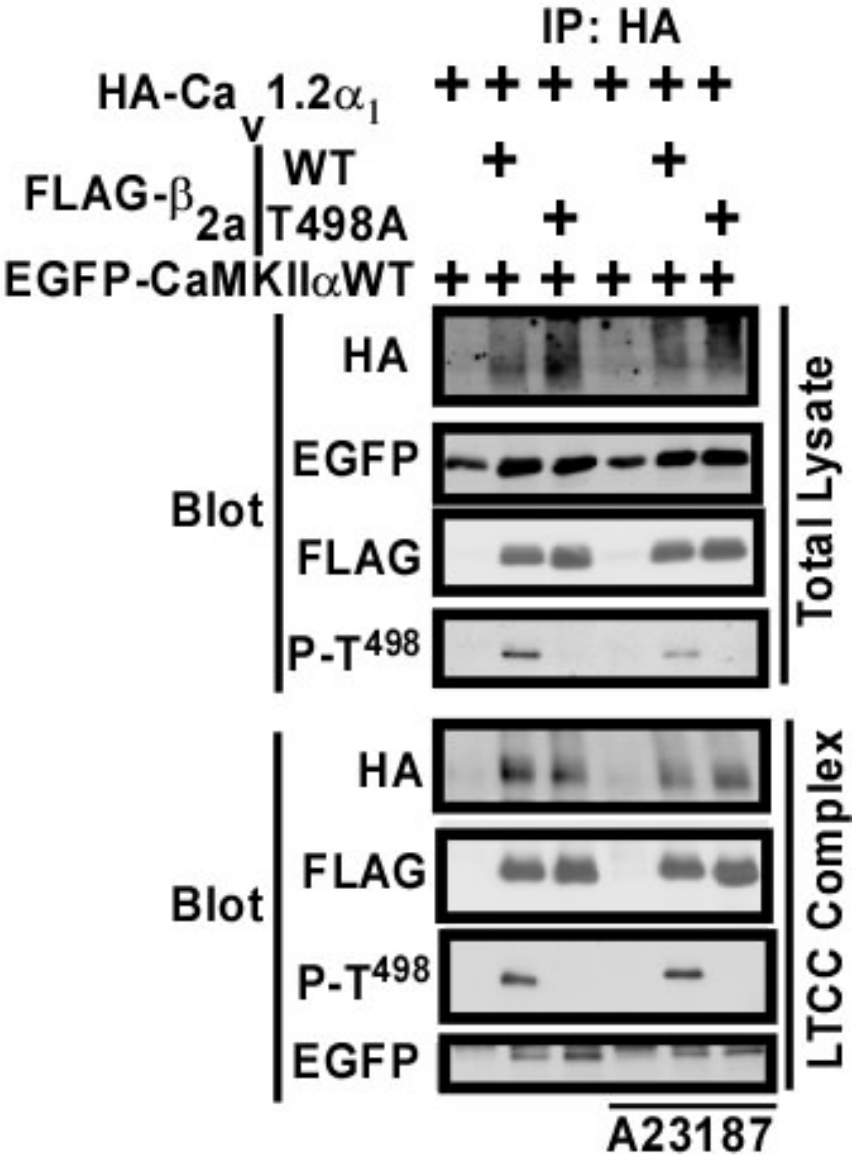

Figure 25. Validation and Specificity of P-T286 CaMKII antibody.
HEK293 cells co-expressing EGFP-CaMKIIα, HA-Ca$_v$1.2 α_1 and/or FLAG-β_{2a} (WT or T498A) were incubated with or without A23187 (10 μM) and immunoprecipitated using anti-HA antibodies. The immune complexes were analyzed by western blotting as indicated. Antibody dilutions: Anti-HA (1:2000), Anti-FLAG (1:5000), Anti-GFP (1:2000), Anti-phopho-Thr^{286} CaMKII (1:2000)

activated by Ca^{2+} ionophore (For example **Figure 25 and 28**). Secondly, the robust phospho-Thr286 antibody signal detected in lysates and LTCC complexes containing WT β_{2a} subunits was eliminated by mutation of Thr498 in β_{2a} to Ala (**Figures 13A, 25 and A11**). Moreover, a similar robust phosphorylation of WT β_{2a} was detected when EGFP-CaMKIIα was replaced by untagged CaMKIIα or myc-CaMKIIδ which resolve clearly from FLAG-β_{2a} (**Figures 13A, 27, A11 and A12**), and in non-transfected cells (**Figure A9**). Thus, I specifically detected Thr498 phosphorylation of β_{2a} in the LTCC complex under these conditions, and not Thr286 phosphorylation of co-precipitating EGFP-CaMKIIα.

I then examined the importance of binding of CaMKIIα to β_{2a} in Thr498 phosphorylation. Mutation of Leu493 to Ala to interfere with CaMKII targeting to LTCC complexes almost abolished total phosphorylation of β_{2a} at Thr498 (**Figure 26A**) and abrogated Thr498 phosphorylation within LTCC complexes (**Figure 26B**). To determine if the effect of CaMKII binding on β_{2a} was independent of the α_1 subunit I omitted the $Ca_v1.2\alpha_1$ cDNA from the cotransfections. In the binary β_{2a}-EGFP-CaMKIIα context, the effect of binding on Thr498 phosphorylation in total lysates and in β_{2a}-CaMKII complexes was similar to that in the ternary context (**Figure A10**). To rule out any artifacts on the phosphorylation of Thr498 due to the EGFP-tag, I showed that phosphorylation of β_{2a} coexpressed with untagged CaMKII was also reduced by the L493A mutation (**Figure 27**).

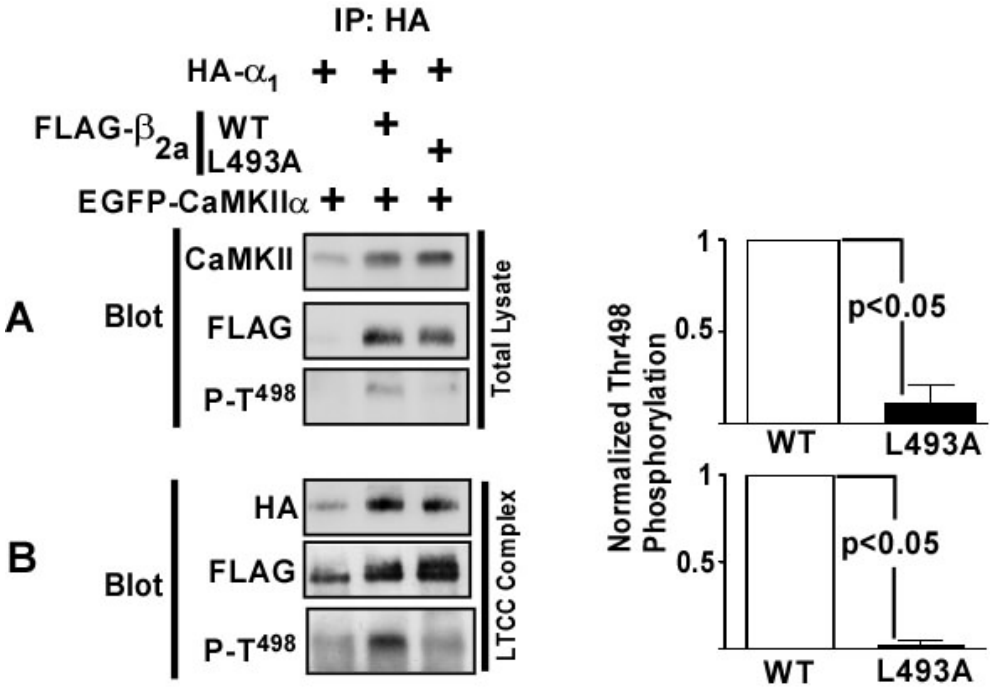

Figure 26. CaMKIIα binding enhances β_{2a} phosphorylation at Thr498 in Ternary LTCC complexes.

Lysates of HEK293 cells co-expressing EGFP-CaMKIIα, HA-$Ca_v1.2$ α_1 and/or FLAG-β_{2a} (WT or L493A), as indicated, were immunoprecipitated using anti-HA antibodies. The total lysates (**A**) and immune complexes (**B**) were analyzed by western blotting as indicated. Phosphorylation of wild-type and mutated FLAG-β_{2a} proteins at Thr498 was quantified. Phosphorylation of FLAG- β_{2a}(L493A) was normalized to that of wild-type β_{2a} and the mean±sem (n=3) is plotted: a one sample t-test was used for statistical comparison. Anti-HA (1:2000), Anti-FLAG (1:5000), Anti-GFP (1:2000), Anti-phopho-Thr^{286} CaMKII (1:2000)

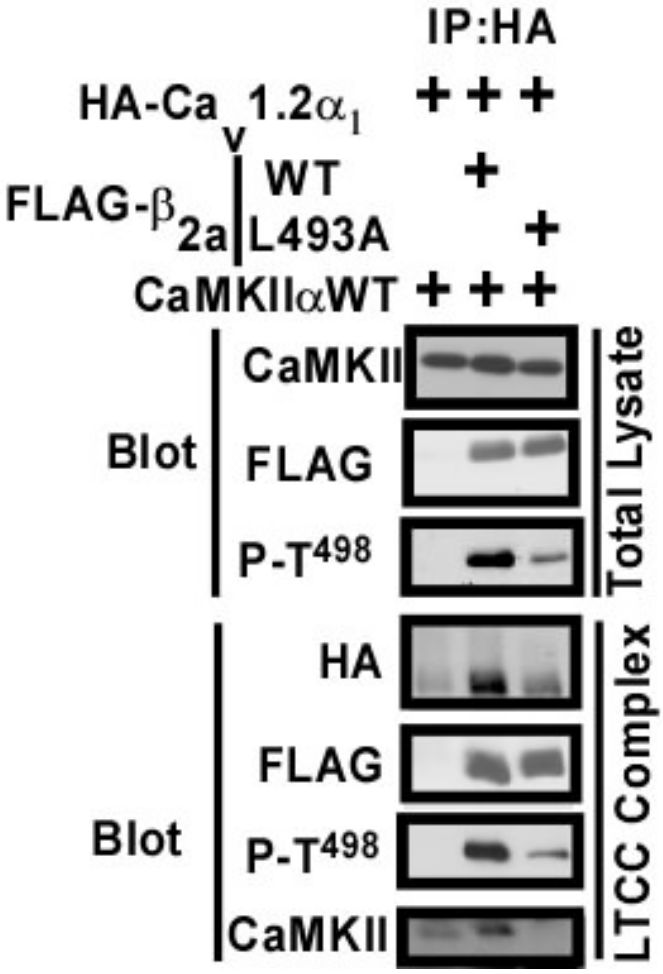

Figure 27. Binding of untagged CaMKIIα enhances β_{2a} phosphorylation at Thr498 in ternary LTCC complexes.
Lysates of HEK293 cells co-expressing untagged CaMKIIα, HA-$Ca_v1.2$ α_1 and/or FLAG-β_{2a} (WT or L493A), as indicated, were immunoprecipitated using anti-HA antibodies. The total lysates and immune complexes were analyzed by western blotting as indicated.

Since CaMKII activation increases the efficiency of phosphorylation and enhances binding to β_{2a}, I tested if stimulation of Ca^{2+} entry to activate CaMKII may overcome the loss of Thr498 phosphorylation due to the L493A mutation. The Ca^{2+} ionophore A23187 did not alter the effect of Leu493 Ala mutation on Thr498 phosphorylation (**Figure 28A**). Notably, constitutively active EGFP-CaMKIIα (T286D), was unable to reverse the loss of β_{2a} Thr498 phosphorylation due to loss of CaMKII binding (**Figure 28B**). Collectively, these data show that CaMKII binding to β_{2a} is required for efficient phosphorylation of a key regulatory site (Thr498) in β_{2a} without affecting overall phosphorylation of multiple sites in the $Ca_V1.2$ α_1 or β_{2a} subunits.

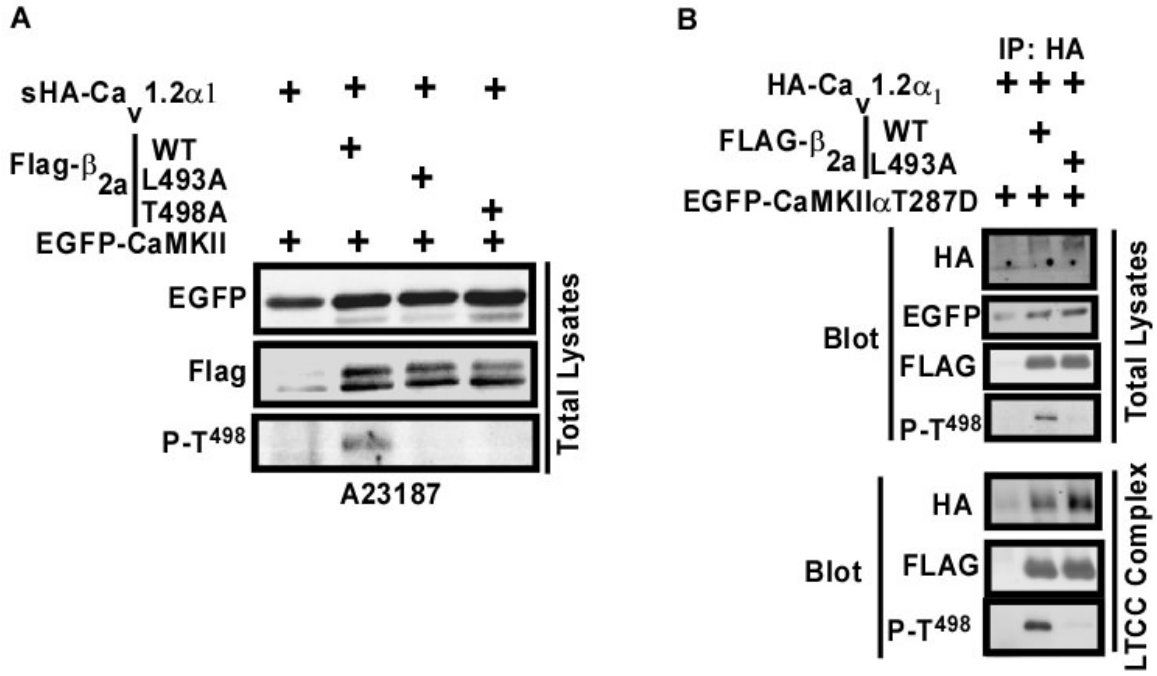

Figure 28. Activation of Ca^{2+} entry or CaMKII does not affect the enhancement β_{2a} phosphorylation at Thr498 by CaMKII binding.
(**A**) Lysates of HEK293 cells co-expressing EGFP-CaMKIIα, HA-$Ca_v1.2$ α_1 and/or FLAG-β_{2a} (WT, T498A or L493A), as indicated. Following a 5-minute treatment with 10μM A23187, the cells were lysed and the extracts analyzed by western blotting for Thr498 phosphorylation. This blot of representative of 3 similar experiments.

(**B**) Lysates of HEK293 cells co-expressing EGFP-CaMKIIα (T286D), HA-$Ca_v1.2$ α_1 and/or FLAG-β_{2a} (WT or L493A), as indicated. The cells were lysed and the extracts and anti-HA immunoprecipitates analyzed by western blotting for Thr498 phosphorylation.

3.4 DISCUSSION

LTCC facilitation by CaMKII can be important for physiological augmentation of cellular Ca^{2+} signals in response to hormones or growth factors [38, 97]. Similarly, hyper-activation of $Ca_V1.2$ LTCCs by CaMKII is implicated in Timothy Syndrome, a multi-organ human genetic disorder whose symptoms include mental retardation and cardiac disease[17, 52, 61, 162]. Furthermore, the loss of dendritic spines from striatal medium spiny neurons following dopamine depletion in animal models of parkinsonism results from excessive $Ca_V1.3$ LTCC activation [40] in parallel with CaMKII over-activation[123]. Thus, understanding mechanistic interactions between CaMKII and LTCCs promises to provide insights into physiological and pathological processes in multiple tissues.

CaMKII targeting to neuronal LTCCs by the β subunits

Subcellular compartmentalization is emerging as a key feature of Ca^{2+} signaling[18]. Colocalization and functional studies suggest that a proportion of CaMKII and LTCCs exist in the same subcellular compartments[33, 34, 38, 79, 97, 146] and CaMKII association with the LTCC complex appears to be essential for facilitation of the channels[63, 140, 144, 147] (**see Introduction**). Even though the molecular basis for CaMKII association with LTCCs and the role of this association in LTCC regulation is poorly understood, it is widely recognized that the β subunits are important in organizing signaling complexes at the channel[25, 27, 47].

Multiple forms of β subunit and CaMKII-dependent LTCC facilitation have been observed in heterologous cells[63, 140] or reconstituted in isolated membrane

patches[79, 144]. This implies that core elements for CaMKII regulation of $Ca_V1.2$ LTCCs in cardiomyocytes are intrinsic to the channel and must exist in neurons. CaMKII was previously shown to co-immunoprecipitate with cardiac $Ca_V1.2$ LTCC complexes. The present findings are the first to show that CaMKII associates with $Ca_V1.2$ LTCCs in forebrain.

Based on our studies, β subunit identity appears to control association of CaMKII with LTCCs in forebrain. Although $Ca_V1.2\alpha_1$ subunits were similarly enriched in both β_1 and β_4 subunit immune complexes, CaMKII was only detected in the β_1 subunit complex (**Figure 16**). Our biochemical studies suggest that CaMKII will also associate with neuronal LTCCs containing the β_1 or β_2 subunits, but not associate with those containing the β_3 or β_4 subunit (**Figures 16, 17 and 21**). This selective association with $Ca_V1.2$ LTCCs containing the β_1 and β_2 subunit variants appears to be due to differential scaffolding/adaptor protein functions of the β subunits. The GK domain of the β subunits interacts with the AID of the α_1 subunit, creating a stable scaffold for the C-terminal domain conserved in β_{1b} or β_{2a}, but not in β_3 or β_4, to anchor CaMKII. The number and stability of CaMKII-LTCC complexes may be influenced by additional interactions, including membrane association of β_1 or the β_2 subunits via N-terminal lipid modifications (reviewed in [25, 167]), additional interactions of β subunits with N- and C-terminal domains of the $Ca_v1.2\alpha_1$ subunit[29, 157, 168, 169] and CaMKII interaction with other $Ca_v1.2\alpha_1$ binding partners such as α-actinin[151, 170]. These additional factors may contribute to the weak targeting of β_{1b} and CaMKII to the $Ca_v1.2\alpha_1$ subunits in our HEK293 studies(**Figure 18**). However, the GK-AID interaction appears to be fundamental for creation of a stable scaffold that specifically targets CaMKII to LTCCs containing the β_{1b} or β_{2a} subunit.

Association of β subunits with LTCCs in brain

Differential functions of β subunit isoforms in controlling the activity and membrane expression of VGCCs are widely accepted[25, 27, 47]. These differences are emphasized by the embryonic lethality of global knockout of β_1 and β_2 subunit genes in mice [171, 172], whereas animals lacking the β_3 and β_4 subunits are viable[173, 174]. Relative expression levels of the β subunit variants are thought to play a major role in determining the specificity of β subunit association with LTCC α_1 subunits [25, 45, 46, 175, 176]. In contrast to the predominant expression of LTCC β_2 isoforms in cardiomyocytes, a mixture of β isoforms are differentially expressed in a complex, developmentally-regulated and cell-specific manner in the brain. The CaMKII-binding β_1 and β_2 subunits are expressed mainly in hippocampus, striatum, cerebellum and photoreceptors[44-46, 177] and predominate during early postnatal development in rodents[25, 45]. However, by adulthood, the β_3 and β_4 subunit variants together comprise about 70% of total β subunits in forebrain[44-46]. Thus, CaMKII association with neuronal $Ca_V1.2$ LTCCs may be more prevalent during postnatal development and only in certain brain regions or cell types.

CaMKII anchoring and LTCC regulation

It is increasingly apparent that subcellular targeting of CaMKII via protein-protein interactions promotes the phosphorylation of specific substrate targets in intact cells[159, 160]. We previously reported that CaMKII-dependent facilitation of $Ca_V1.2$ LTCCs requires the phosphorylation of β_{2a} subunits at Thr498, and showed that CaMKII binds with high affinity ($K_d \approx 100$ nM CaMKII subunit) to the β_{1b} and β_{2a} subunits, but not the β_3 or β_4 subunits. However, the relationship(s) between protein-protein interactions, phosphorylation and facilitation was not investigated. The primarily β-subunit-dependent association of CaMKII with

LTCCs may facilitate CaMKII interaction with additional domains on the α_1 subunit[147], and may enhance CaMKII phosphorylation of functionally relevant sites, such as Ser1512 or Ser1570 in the α_1 subunit[63] or Thr498 in the β_{2a} subunit[79]. It may initially seem surprising that total phosphorylation of LTCC subunits was not affected by co-expression of EGFP-CaMKII or by L493A mutation of β_{2a}, even on the significant background of substantial LTCC subunit phosphorylation by endogenous kinases (**Figures 23 and 24**). However, it is important to note that these experiments were performed under basal cell incubation conditions in which CaMKII is only partially active. The partially active CaMKII may be highly selective for the preferred Thr498 site in β_{2a} in comparison to other sites that can be phosphorylated *in vitro* by fully activated kinase[79]. Indeed, we directly demonstrated that CaMKII binding facilitates Thr498 phosphorylation in β_{2a} subunits within LTCC complexes isolated by immunoprecipitation. Even though contributions of other kinases such as PKC and PKA to the phosphorylation at Thr498 cannot be entirely ruled out, our data strongly suggest that CaMKII is responsible for the changes in Thr498 phosphorylation. First, the enhancement of Thr498 phosphorylation by CaMKII co-transfection in a subset of experiments (**For example, Figure A10**) indicates direct Thr498 phosphorylation by CaMKII. Second, the dependence of Thr498 phosphorylation on CaMKII binding is only robust when exogenous CaMKII is co-transfected with LTCCs (compare **Figures 26 and A9**). Since phosphorylation of β_{2a} at Thr498 phosphorylation promotes CaMKII-dependent LTCC facilitation the present findings provide a biochemical basis for the recently LTCC facilitation that required CaMKII binding[178].

My data in intact cells contrast somewhat what previous observations by Dr. Chad Grueter in this lab. He did not detect a significant effect of the L493A

mutation on Thr498 phosphorylation *in vitro*. Perhaps in a well mixed, *in vitro* system, where CaMKII can readily access the β_{2a}, stable association of the proteins is not essential for efficient Thr498 phosphorylation. On the other hand, in the cytoplasm of intact cells, protein movements may be more restricted, and thus necessitate stable CaMKII/LTCC colocalization for efficient Thr498 phosphorylation. However, it is also possible that in intact cells CaMKIIα phosphorylates Thr498 with similar efficiency in β_{2a} (WT) and β_{2a} (L493A) but cellular phosphatases more efficiently dephosphorylate phospho-Thr498 in the β_{2a} (L493A) than in WT protein because binding of CaMKII to the WT protein protects phospho-Thr^{498}. The *in vitro* assays had no phosphatases to create such a difference in Thr498 phosphorylation in the two proteins. Thus, within cells the relative localization of LTCCs, CaMKII and phosphatases may critically control access to Thr498 and LTCC facilitation.

Overall, these studies have demonstrated the importance of β subunits in selective targeting of CaMKII to phosphorylate a regulatory site of LTCCs, providing a biochemical explanation for regulation of LTCCs and perhaps of other VGCCs by CaMKII.

CHAPTER IV

OVERALL DISCUSSION AND FUTURE STUDIES

This thesis explored the molecular mechanism of LTCC regulation by CaMKII and revealed a strong, regulated, binary interaction of CaMKII and β subunits that allows dynamic tethering of CaMKII to enhance regulatory phosphorylation of the channel. Here I summarize this mechanism and address additional outstanding questions that are important for a more complete understanding of LTCC regulation and function in cardiomyocytes and neurons.

4.1 MECHANISM OF LTCC REGULATION BY CAMKII

Data emerging over the last four years suggest that mechanisms underlying CaMKII modulation of LTCCs are complex and involve both the α_1 and β subunits of LTCCs (**Figure 29**). Binding and phosphorylation of the α_1 subunit has been implicated in enhancement of LTCC currents (**See Introduction**). While most studies of the mechanism of CaMKII regulation have targeted the α_1 subunit, our group examined the role of the β subunit in the CaMKII-dependent regulation of LTCCs. We showed that phosphorylation at Thr498 in β_{2a} is required for CaMKII to increase channel open probability at the single channel level and to facilitate whole cell Ca^{2+} currents in adult cardiomyocytes[79]. My thesis has extended these original observations by showing that β_{1b} and β_{2a} subunits of LTCC dynamically associate with the CaMKII (**Chapter 2**). The binary β subunit/CaMKII

interaction is important for normal targeting of CaMKII to $Ca_v1.2\alpha_1$ subunits (and to $Ca_v1.3\alpha_1$ I/II linker), and for regulatory phosphorylation of β_{2a} at Thr498 in LTCC complexes (**Chapter 3**). Thus, my studies provide insights to reconcile the various mechanisms of LTCC regulation by CaMKII and establish a molecular basis for understanding β subunit regulation of LTCC function in heart, brain and perhaps other tissues.

Model for CaMKII dependent LTCC regulation: My findings are consistent with the following basic model of LTCC regulation by CaMKII (**Figure 29**). At resting conditions, cytosolic Ca^{2+} is low, CaMKII is weakly activated and phosphorylation of β_{2a} at Thr498 is weak, resulting in basal LTCCs activity. Ca^{2+} entry and CaMKII activation enhances binding of CaMKII to the LTCC β subunit, induces rapid Thr498 phosphorylation by the bound CaMKII holoenzyme, which in turn initiates CaMKII dissociation. The facilitation mechanism may then be turned off by dephosphorylation of β_{2a} and CaMKII to reset the channel to basal state. It is yet unclear which of these steps—binding, Thr498 phosphorylation or dissociation—triggers the transition of the channel to facilitated states. Some of the lack of clarity exists because of two key observations we have made. First, the open probability of LTCCs containing the β_{2a}(Thr498Glu) mutant (which mimics T498 phosphorylation and prevents CaMKII association with LTCC, **Figures 12, 13, A13 and A14**) is indistinguishable from the WT[179], suggest that Thr498 phosphorylation is insufficient for facilitation of LTCC currents. However, it goes without saying that mutation of Thr498 to Glu may not fully

mimic phosphorylation of the residue. Secondly, LTCCs containing β_{2a}(Thr498Ala) mutant (which bind CaMKII, **Figures 12, 13, A12, A13 and A14**) are not facilitated by CaMKII, implying that CaMKII binding is insufficient for facilitation. These Thr498Glu and Thr498Ala phenotypes suggest that facilitation may actually depend on precise coordination of the binding and phosphorylation events. If so, Thr498 phosphorylation may then be a bifunctional switch to control facilitation: it may couple CaMKII binding to β_{2a} to phosphorylation of sites on the α_1 subunit known to be essential for facilitation (**see Introduction**) but eventually discontinue CaMKII signaling by dissociating the active CaMKII or preventing rebinding of CaMKII to the channel. The true picture of LTCC facilitation is likely to be even more complicated, particularly if one takes into account the multiple alternative interpretations of events downstream of Thr498 phosphorylation, such as kinase disinhibition, phosphorylation of other sites on β_{2a} and structural rearrangements of the channel complex (**see discussion of Chapter 2**).

The structural mechanism of LTCC regulation by CaMKII mentioned in **Chapter 2** raises the fundamental questions of how CaMKII binding, dissociation or LTCC phosphorylation alters channel activity. An extensive theory on the structural basis of LTCC regulation by CaMKII is premature but I want to discuss the subject briefly, drawing on insights from a few prior studies. The IS6-AID domain of the α_1 subunit (**see Introduction**) forms a rigid structure that connects and properly orients the β subunit to the pore in the α_1 subunit[55, 180]. This rigid

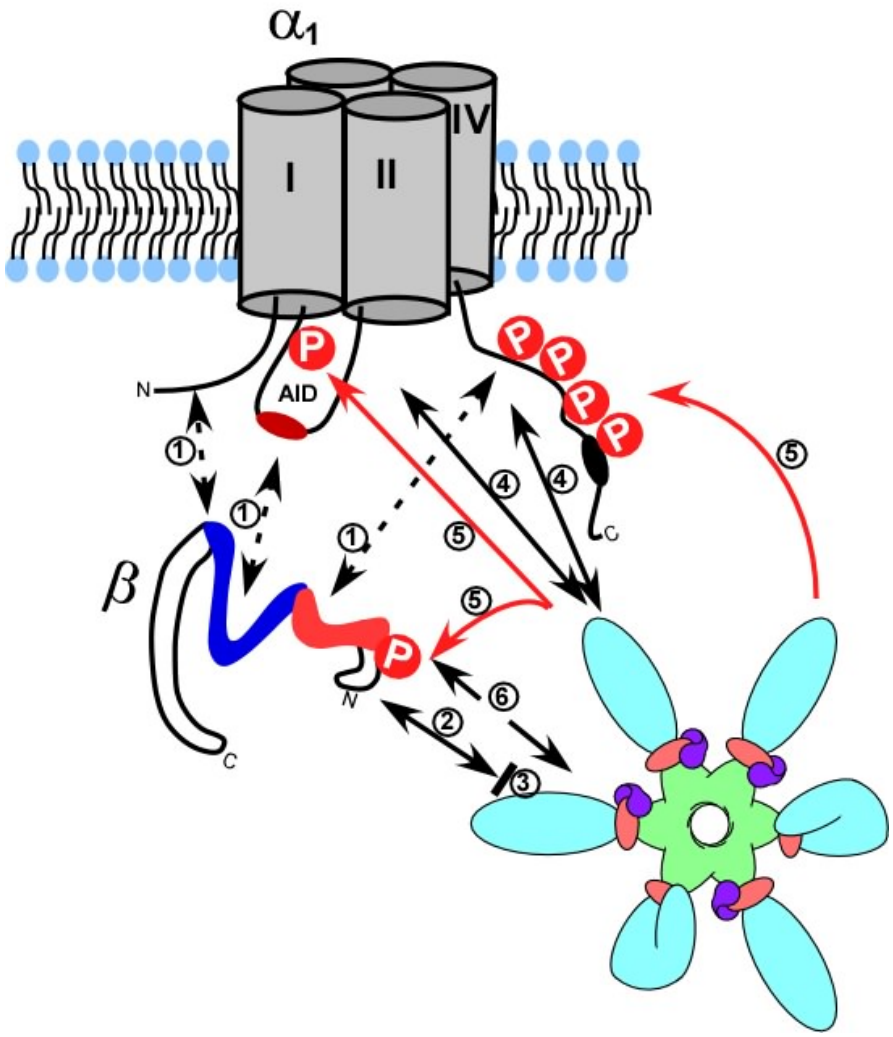

Figure 29. A model for β_{2a}-dependent regulation of LTCCs by CaMKII
Solid arrows indicate CaMKII binding and dashed arrows indicate β subunit binding. Multiple interactions (**1**) with α_1 subunit target β subunits to LTCCs . Activated CaMKII binds β_{2a} (**2**) to form a ternary complex (CaMKII-β_{2a}-α_1) and the resulting inhibition of bound CaMKII (**3**) stabilizes the kinase at the channel. The increased local CaMKII concentrations may enhance CaMKII binding to α_1 (**4**) and/or phosphorylation of β_{2a} at Thr498, of α_1 at S1517 or S1575 (or S439 in the Timothy Syndrome mutant) (**5**), resulting in LTCC facilitation. Phosphorylation of β_{2a} at T498 releases CaMKII (**6**) to further increase phosphorylation of the α_1 subunit and thus promote CaMKII-dependent LTCC facilitation, or simply exposes Thr 498 to allow phosphatases to terminate CaMKII actions.

structure of the IS6-AID is essential for normal coupling of β subunit/CaM-dependent processes to the channel pore during voltage or Ca^{2+}-dependent regulation and could be altered by CaMKII binding. The β subunit also links the IS6-AID and the channel pore to the C-terminal tail of the α_1 subunit, which contains both the CB/IQ region involved in CaM-dependent regulation and an autoinhibitory region[29, 83, 157, 181]. Thus, the β subunit is a central structural coordinator of LTCC during regulation and a potential locus for structural effects of CaMKII. The association of a bulky CaMKII (~200 Å diameter) [90, 182] with the cytosolic face of the LTCC complex and pore (~115Å combined diameter) [90, 182] may perturb the link between the β subunit, the α_1 C-terminus, the AID and the pore. In this way, CaMKII binding can potentially inhibit the opening and closing of the LTCC pore. Thr498 phosphorylation may be required to 'free' the channel pore from such steric hindrance by destabilizing the LTCC-CaMKII complex. Of course, it is also possible that CaMKII binding primes the channel so that Thr498 phosphorylation can more efficiently switch it to facilitated states. However, the effect of β subunit phosphorylation at Thr498 on LTCC opening may not be purely structural and direct. The phosphorylation may dissociate CaMKII to enhance phosphorylation of other sites on the α_1 and β to in a way that perturbs coordination of LTCC domains by β subunits. For example, Thr498 phosphorylation may allow preassociated CaMKII to phosphorylate the aforementioned IS6-AID (for example Ser439 in the TS mutant) to alter coupling of the β subunit to the channel pore, resulting in suppression of LTCC inactivation in TS mutants[162]. In summary, even though the structural basis of

CaMKII/β-dependent LTCC regulation is still unknown, my thesis research has provided useful insights to help guide future mechanistic and structural studies.

4.2 OUTSTANDING QUESTIONS AND FUTURE DIRECTIONS

Despite the insights provided by my studies, several key questions related to the importance and dynamics of CaMKII/β-dependent LTCC regulation remain unresolved. In this section I address some of these questions.

a. What is the functional relevance of β subunit-dependent CaMKII targeting to LTCCs?

At a *biophysical level*, CaMKII targeting to LTCCs by the β subunit enhances open probability of single LTCCs and increases whole-cell LTCC currents in cardiomyocytes[178]. The CaMKII binding-dependent Thr498 phosphorylation reported herein appears to be an intermediate step in the β_{2a}-dependent LTCC regulation. It will be important to compliment the biophysical studies of LTCCs with studies that examine binding and phosphorylation of specific sites on the α_1 previously known to be involved in CaMKII-dependent regulation. Overall, the biophysical findings and my biochemical data are consistent with a role of β subunit-dependent targeting in the tightly controlled changes in Ca^{2+} are essential for many normal processes in cardiomyocytes and neurons (**see Introduction**).

In cardiomyocytes, the LTCC complex is a preferred entry point for the Ca^{2+} that triggers release of Ca^{2+} through ryanodine receptors to control multiple aspects of Ca^{2+} signaling (such as LTCC facilitation) and homeostasis[183]. Cardiomyocyte

LTCC facilitation by CaMKII augments cellular Ca^{2+} signals in response to hormones[38, 97] or repeated depolarizations[79]. Cardiomyocyte Ca^{2+} augmentation is a hallmark of LTCC-dependent EC-coupling, which is known to be regulated by CaMKII-binding β_1 and β_2 isoforms[171, 172]. Animals lacking the β_2 subunit of LTCCs exhibit multiple abnormalities including impaired excitation-contraction coupling in cardiomyocytes, and ultimately die prenatally due to inability of their hearts to develop[172]. In isolated myocytes excitation-contraction coupling is dependent on CaMKII binding to β_{2a} [178]. Even though the β_2 isoform predominates in cardiomyocytes, other β isoforms are also present[26, 43] and may play analogous roles, especially the β_{1b}, which binds CaMKII and regulates excitation contraction-coupling. Thus, the β_1, β_2 and CaMKII all share control of excitation-contraction coupling, which is consistent with their involvement in a common LTCC regulation pathway.

Even though CaMKII-dependent LTCC facilitation has not been reported in *neurons,* for various reasons the mechanisms that underlie CaMKII-dependent cardiac LTCC facilitation appear to be preserved in neurons. First, multiple types of CaMKII-dependent LTCC facilitation have been observed in heterologous cells[63, 140] or reconstituted in isolated membrane patches[79, 144], implying that core elements of the process are intrinsic to the channel and exist in multiple cell types. Secondly, Ca^{2+} release from intracellular stores through ryanodine receptors is essential for the CaMKII-dependent LTCC facilitation in cardiomyocytes[184] and in neurons LTCCs are also functionally coupled to

ryanodine receptors of intracellular Ca^{2+} stores[185]. Thus, the mechanisms that underlie CaMKII-dependent cardiac LTCC facilitation may also contribute to LTCC facilitation in neurons, and may provide the Ca^{2+} influx for key neuronal processes such as presynaptic potentiation following repeated stimulation of presynaptic terminals in the brainstem[186, 187] and cerebellum[188]. The presynaptic potentiation is consistent with the presence of local Ca^{2+}-dependent feedback mechanisms that physiologically augment Ca^{2+} entry to regulate neurotransmitter release[48]. Furthermore, a functional link between closely positioned CaMKII and LTCCs triggers excitation-transcription coupling in neurons[189] which may be associated with enhanced phosphorylation of the channel's α_1 and β subunits (**see Introduction**).

In contrast to myocytes, a heterogeneous mixture of the β subunits is expressed in neurons, suggesting that CaMKII-dependent LTCC regulation varies from neuron to neuron. For example, CaMKII is likely to regulate LTCCs in neurons expressing predominantly the β_1 or β_2 but not in neurons expressing mainly the β_3 or β_4 isoform because binding and phosphorylation of the conserved Thr498 would be impaired in β_3 or β_4. Thus, future experiments to determine LTCC facilitation by CaMKII in neurons may require careful consideration of the identity of the β subunit in the neuronal subtype. Indirect insights into LTCC regulation in neurons by β subunits have come from manipulations of VGCC β subunit expression in LTCC-dependent neuronal processes. For example, by genetically deleting the β_2 subunits, three groups have implicated β_2 in regulating

neurotransmission at photoreceptor ribbon synapses, in transmission and exocytosis by inner hair cells and in hearing[190, 191]. Furthermore, β_2 -containing synapses of hippocampal neurons maintain a higher probability of release than β_4 containing synapses, which is reminiscent of enhanced basal VGCC tone[192]. Given the presynaptic roles of CaMKII (**see Introduction**), differential presynaptic CaMKII targeting by the β isoforms may alter LTCC or VGCC function in neurons.

Deletions of the β subunits that do not bind CaMKII, i.e β_3 and β_4, resulted in a variety of deficits that are distinct from effects of β_2 deletions. Loss of β_4 due to a spontaneous mutation is not lethal but produces an epileptic, ataxic neurological phenotype[25], suggesting that β_4 is important in controlling excitability in some important neuronal circuits. β_3 knockout animals show resistance to pain sensation[173]. These phenotypes are consistent with the existence unique roles of CaMKII targeting in physiological functions. Whether the functional distinctions between β_2 subunits and β_3 or β_4 are due to differences in CaMKII-targeting to LTCCs or other VGCCs is an interesting question that remains to be adressed.

In addition to highlighting β subunit isoform differences, my preliminary studies have also raised the potentially important CaMKII isoform-dependent differences in LTCC regulation in various tissues. In contrast to CaMKIIα, cotransfection of the CaMKIIδ isoforms (WT or constitutively active) resulted in Thr498 phosphorylation that was virtually independent of β_{2a} binding (**Figure 13, A11**

and A12). The distinct effects of CaMKIIα and CaMKIIδ isoforms may reflect differences in their activation state and strength of interaction with the β subunit or other proteins, and will best be analyzed by comparing their binding to, and phosphorylation of LTCCs side-by-side. HEK293 cells also appear to express a low level of at least one endogenous CaMKII isoform of unknown identity[193, 194]. In cells that were not transfected with CaMKII, mutation of β_{2a} to disrupt binding of CaMKII reduced phosphorylation of β_{2a} at Thr498 by 60% in total lysates but had no effect on Thr498 phosphorylation in immunoprecipitated LTCC complexes (**Figure A9**). These results suggest binding of the endogenous CaMKII isoform in HEK293 cells to LTCC is neither required nor sufficient to alter phosphorylation of Th498 within LTCC complexes, and only mildly modulates phosphorylation of total β_{2a} at Thr498. These findings contrast starkly with the findings in cells overexpressing CaMKIIα (**Figures 26 and 27**) where binding robustly increased Thr498 phosphorylation. The effects of endogenous kinase resembled those of δ isoforms of CaMKII, suggesting HEK cells may endogenously express the CaMKIIδ. Nevertheless, I have not demonstrated in these studies that endogenous Thr498 phosphorylation is due to actions of CaMKII but not other kinases.

b. Is there a role of the β subunit in pathophysiological regulation of LTCCs by CaMKII?

CaMKII/LTCC-dependent pathophysiology. There is growing evidence supporting mutual reinforcement of LTCC and CaMKII activity in a positive feedback loop[24, 34, 147, 189] in both cardiomyocytes and neurons. The vicious cycle of LTCC facilitation and CaMKII activation may lead to cardiac (e.g Timothy Syndrome) and neurological pathologies (e.g Parkinson's Disease) linked to

excessive LTCC activity, Ca^{2+} entry, and CaMKII activation[17, 40, 52, 61, 123, 183, 195] (**see Introduction and Chapter 3**). The LTCC α_1, β/CaMKII complex provides a suitable mechanism to set up the perpetual Ca^{2+} overload needed to cause disease.

Pathological role of β/CaMKII-dependent facilitation in heart: Multiple reports suggest that the mechanisms that enable the β subunit of LTCC to facilitate the channels in normal circumstances may be engaged in disease. Elevated levels of β_{2a} have been detected in failing human hearts[196] and may contribute to channel hyperactivity and arrhythmia-generating electrical abnormalities and cell-death[197-199]. Furthermore, overexpression of exogenous β_{2a} in adult cardiomyocytes increases LTCC currents, and induces overload of SR Ca^{2+}, and cell-death[149, 178]. The cell-death occurs via a CaMKII-dependent pathway that requires CaMKII binding to the β subunit and Thr498 phosphorylation[178]. As CaMKII levels and activity are also elevated in failing mouse hearts[109, 110], the β_{2a}-CaMKII pathway seems to be an important in the pathogenesis of heart. My studies support and complement these findings by providing the biochemical link between CaMKII binding and Thr498 phosphorylation in LTCC complexes.

Pathological role of β/CaMKII-dependent facilitation in the brain. The role β/CaMKII-dependent LTCC regulation in neurological disease has not been explored but multiple studies provide some initial insights. Four key properties of LTCCs—their expression density[200], phosphorylation[201], open

probability[200]and whole-cell currents[202, 203]. The aging-related pathology in brain bares the cellular, biochemical and elephysiological hallmarks of the β/CaMKII-dependent LTCC regulation in myocytes (**see Introduction**) and may therefore also be a major contributing factor in diseases associated with aging. Indeed, the enhancement of LTCC function in neurons is associated, at least in some cases, with neuropathology such as reduced survival of the hippocampal neurons[204], age-related learning deficits[202, 203] and loss of dendritic spines in experimental Parkinsons Disease model animals[40]. It will be interesting to determine if β_1 and β_2 subunits alter CaMKII-dependent phosphorylation and regulation of LTCCs in the Parkinson's Disease models.

Targeting CaMKII-LTCC interaction for therapy: CaMKII and β subunits have individually been explored as molecular therapeutic targets for cardiac disease but their interaction has not been reported as a potential drug target. Inhibition of CaMKII is effective in reducing arrhythmia-inducing early-afterdepolarizations in cardiomyocytes[162, 178] and in mitigating structural damage to heart following myocardial infarctions[110]. Similarly, structural damage to the heart is attenuated by gene therapy that utilizes *in vivo* knockdown of endogenous β_{2a}[205] and fragments of β_{2a} reduce Ca^{2+} overload[206, 207]. These manipulations of the β subunit have focused on the modulation of α_1 expression by β_{2a}, which may produce undesirable side effects associated with a lack of the normal number of functional channels at plasma membranes. Since LTCC-dependent cardiomyocyte dysfunction can be blocked by preventing

phosphorylation of β_{2a} at Th498 or by disruption of CaMKII binding to β_{2a} [178], interfering with CaMKII binding to or phosphorylation of β_{2a} is an attractive option for therapeutic intervention. In practice, β subunits that lack the C-terminal CaMKII binding domain could be introduced into cells to mitigate the deleterious effects of CaMKII binding. Alternatively, peptides that selectively interfere with CaMKII binding to β_{2a} but not its activity or normal functions could be introduced into ailing cells. Such approaches would be aided by a precisely defined mechanism and information on dynamics of CaMKII binding and phosphorylation of Thr498. My biochemical studies provide a good starting point for obtaining such information but new methods of studying binding and phosphorylation of β subunits *in vitro* and in cells will be essential.

c. How fast do LTCC-CaMKII complexes assemble and dissociate?

LTCC regulation occurs on the milliseconds to seconds timescale and underlies the long-term dynamic control of many processes including the strength and rate of heart contraction, neurotransmission and neuronal plasticity. As indicated in the model in **Figure 29**, one could envisage that CaMKII binds to β_{2a} and then phosphorylates Thr498, creating a timed feedback switch to dissociate CaMKII and terminate its facilitation of LTCCs. I began addressing the dynamic regulation of CaMKII-LTCC complexes by monitoring their dissociation *in vitro* (**Figures 16 and A15**). However, these binding studies, like other CaMKII binding studies in this thesis, rely on slow conventional approaches (e.g., plate assays, overlays, cosedimentation assays) that provide little information about

association and dissociation rates, or true equilibrium interactions. Thus, development of assays that monitor CaMKII-β_{2a} interactions on a shorter timescale in real time is likely to provide fresh insight into their physiological relevance and regulation.

Dynamic assembly and regulation of LTCC-CaMKII complexes in vitro: Fluorescence anisotropy is a common in-solution assay used to determine association and dissociation rates and equilibrium binding constants *in vitro*[208]. Anisotropy measurements may provide important new information about association/dissociation rates, binding affinities, and Ca^{2+} concentration dependence that will provide far greater insight into likely cellular dynamics of these complexes. Thus application of fluorescence anisotropy may reveal more precise answers to questions such as: is the Ca^{2+} sensitivity appropriate for complex formation at the local Ca^{2+} concentrations that are thought to be generated in the vicinity of the LTCC complex? Does Thr286/7 autophosphorylation enhance the Ca^{2+} sensitivity so that complexes are more likely to form under physiological conditions? Even greater temporal-sensitivity would be achieved using a stopped-flow system with more rapid mixing of reagents.

Dynamic regulation of LTCC-CaMKII complexes in cells: My preliminary studies show that CaMKII colocalizes with LTCCs in a manner dependent on activation of Ca^{2+} entry, suggesting the colocalization is a dynamically regulated process

(**Figures A6, A7 and A8**). However, these colocalization studies provide a "snap-shot" of steady-state CaMKII and LTCC localization in fixed cells. An alternative method would involve expressing wild-type or mutant Flag-β_{2a} and fixing the cells at various times after enhancing Ca^{2+} entry via LTCCs using various concentrations of BayK8644 or inhibiting Ca^{2+} entry by replacing extracellular Ca^{2+} with Ba^{2+}. To ultimately monitor dynamics of CaMKII association with LTCCs in living cells (HEK293, neurons or cardiomyocytes) in real time, the relevant proteins can be tagged with complementary fluorescent proteins that can be imaged simultaneously (e.g., RFP and GFP) by confocal microscopy and/or used for fluorescence resonance energy transfer (FRET) (e.g., CFP and YFP). My biochemical experiments facilitate the design and interpretation of these future studies by providing mutant proteins with well-defined changes in their protein-protein interactions.

The spatial resolutions of CaMKII-LTCC interactions may be further improved by combining FRET with Total internal reflection fluorescence (TIRF) microscopy. TIRF microscopy is a useful tool to monitor changes in protein-protein interactions within 200 nm of the plasma membrane. In TIRF microscopy, the exciting light beam is directed at an angle such that it undergoes total internal reflection at the boundary between the cover slide glass medium and aqueous cell medium. A resultant evanescent wave that rapidly dissipates within (100-200 nm) excites the flourophores within its radius. Thus, TIRF microscopy offers a powerful technique to monitor dynamic interactions of proteins at the plasma

membrane. A combination of TIRF microscopy and FRET could also be used to monitor precise spatiotemporal dynamics of interactions following Ca^{2+} entry.

These approaches will not only reveal the dynamic regulation of CaMKII interactions with LTCC subunits but can also provide useful information about the impact of the proteins associated with LTCCs or its vicinity on assembly of the channels with CaMKII. Some of these are structural proteins such as α-actinin, PDZ proteins such as NIL-16 and SHANK, CaBP1 which bind the α_1 subunit and AHNAK that binds the β subunit[24, 209]. Others are signaling proteins such as CaM, PP1, PP2A, which associate with LTCC and are able to modulate the activity of CaMKII and the phosphorylation of the LTCC subunits[24, 87]. There are even channels such as the NMDAR, that are not directly associated with LTCCs, but coexist in dendritic spines and modulate LTCC activity[24]. The NMDARs may control CaMKII activity at LTCCs by competing for CaMKII binding to the β subunits or by trapping CaMKII in an activated state[153]. The combined effect of all these auxiliary proteins may modulate the extent of Thr498 phosphophorylation and therefore fine-tune LTCC facilitation.

d. Are β subunits important in regulation of non-LTCC channels?

The β subunit isoforms associate with most VGCC α_1 subunit variants. Interestingly, CaMKII facilitates $Ca_V1.3$ LTCCs by phosphorylating the α_1 subunit at Ser1486[38] and antagonizes the inactivation of $Ca_v2.1$ P/Q type VGCCs by binding within the α_1 C-terminal tail[210]. It will be important to investigate

whether β subunits also play a role in these CaMKII-dependent effects. Unlike $Ca_v1.3$ and 2.1, $Ca_V3.2$ channels are not generally thought to associate with β subunits. Yet CaMKII enhances the activity of $Ca_v3.2$ T-type VGCCs, apparently via direct interaction of CaMKII with the II/III linker domain[75]. Thus, binding of CaMKII to the VGCC complex appears to be a common feature for CaMKII-dependent feedback regulation of these channels. The β_1 or β_2 subunits may have a preeminent function in some cases, but in other cases direct interactions with the α_1 subunit may play an important role. Moreover, it will be interesting to investigate whether association of CaMKII with LTCCs via selected β subunit variants plays a role in CaMKII-dependent cross talk between LTCCs and R-type channels in dendritic spines[33, 34] and/or in CaMKII activation to mediate downstream signaling to the nucleus[189].

3.3 THESIS SUMMARY

The findings reported here and in other recent papers suggest that feedback regulation of Ca^{2+} influx via VGCCs is precisely controlled in specific subcellular microdomains by multiple mechanisms that allow CaMKII and other Ca^{2+}-dependent signaling proteins to associate with channel subunits. The precise nature of the feedback regulation by CaMKII seems likely to depend on the identity of the β subunit associated with the complex. The regulated interaction of activated CaMKII with β_1 and β_2 variants, and phosphorylation of the subunits at Th498 seem likely to be important for LTCC facilitation, although phosphorylation of β_3 and β_4 may also play a role in some cases. Our findings are in line with recent studies, suggesting that subcellular targeting of CaMKII via its interactions with CaMKAPs modulates the specificity of its downstream actions. These

complex biochemical mechanisms for feedback regulation of Ca^{2+} influx via VGCCs presumably provide great flexibility for modulating a variety of downstream signaling events such as cardiac excitation−contraction and excitation−transcription coupling and neuronal synaptic plasticity. Moreover, alterations in the association of β subunits with VGCCs might disrupt feedback regulation and downstream signaling in heart failure and other diseases.

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APPENDIX

CaMKII catalytic site-binding peptides impair binding of β_{2a}

The conservation of CaMKII binding domains of β_{2a}, NR2B and CaMKII-autoregulatory domain (**Figure 9B**) suggested that they interact with CaMKII by a similar mechanism. I began to narrow down the molecular mechanism of these interactions by investigating the effects of synthetic peptides based on NR2B and CaMKII autoregulatory domain on CaMKII binding to β_{2a}. As a positive control, I showed that His-β_{2a}(410-505) protein inhibited CaMKII binding to β_{2a} (**Figure A1, *Top panel***) in a concentration-dependent manner. A 20-amino acid NR2B peptide (NR2B1290-1309) also inhibited the β_{2a}-CaMKII interaction, but with a weaker potency than the His-β_{2a} (410-505). The NR2B peptide inhibition was specific because it was reduced when an NR2B control peptide (1290-1309R1300Q), which is mutated to disrupt CaMKII binding to NR2B[155], was used(**A1, *Top panel***). Autocamtide-2 (AC-2), a 14-amino acid peptide substrate modeled on the sequence surrounding the T286/7 autophosphorylation site of CaMKII[211], inhibited CaMKII binding to GST-β_{2a} as potently as the NR2B peptide, but also less potently than the His-β_{2a} (410-505). The differences in the potency of CaMKII binding inhibition by the peptides (NR2B peptide and the AC-2) and the 95-amino acid His-β_{2a}(410-505) protein may arise from unique binding and folding modes conferred by the extra amino acids in the β_{2a} protein. In contrast to the NR2B peptide and AC-2, the 14-amino acid syntide-2 peptide

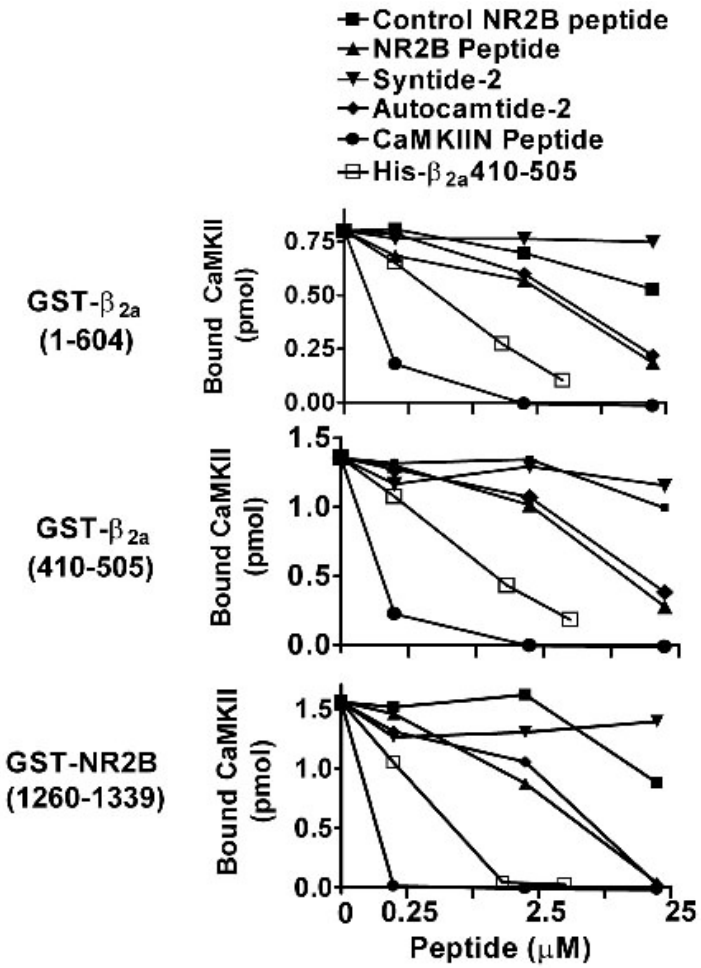

Figure A1. Effect of CaMKII catalytic site binding peptides and proteins on CaMKII binding to β_{2a}.

The indicated concentrations of peptides or proteins and [^{32}P-T^{287}]CaMKIIδ (500 nM) were premixed for 1 hr. CaMKIIδ binding to 5 pmol of immobilized full-length GST-β_{2a} (*top*) or , GST-β_{2a} (410-505, *middle*) or GST-NR2B (1260-1339, *bottom*) in glutathione-coated wells was determined. This set of experiments was performed once.

derived from a phosphorylation site in glycogen synthase binds the CaMKII catalytic site by a mechanism that is distinct from that of AC-2 [212] and thus did not inhibit CaMKII binding to β_{2a} (**Figure A1, Top panel**). These observations suggest that conserved residues surrounding Thr498 in β_{2a}, Thr286 in CaMKII and Ser1303 in NR2B make molecular contacts with a shared set of residues within the CaMKII catalytic domain.

If the 410-505 region is indeed responsible for CaMKII binding to β_{2a} (**Figures 9, 12 and 13**), the NR2B and AC-2 inhibition of CaMKII binding to the full-length subunit should be recapitulated using only the 410-505 domain. As predicted, the inhibition of CaMKII binding to the full-length β_{2a} by NR2B and AC-2 was indistiguishable from the inhibition of binding to GST- β_{2a} (410-505) (**Figure A1, middle panel**). I also performed reciprocal competition assay to confirm that CaMKII binding to the GST-NR2B (1260-1339) was in return inhibited by His-β_{2a}(410-505) (**Figure A1, bottom panel**). This assay was performed in parallel with other peptides, which all inhibited CaMKII binding to NR2B more potently than binding to β_{2a}, for reasons that are not clear. Based on these preliminary competition studies, NR2B and β_{2a} appear to bind CaMKII via a similar mechanism. However, conservation of residues around Ser1303 of NR2B, Thr286 of CaMKII or Thr498 of β_{2a} is not a strict requirement for binding of CaMKII[147, 193]. Thus, binding of proteins and peptides to CaMKII catalytic domain via unconserved residues may still prevent β_{2a}/NR2B to CaMKII. To test this possibility, I used a CaMKII inhibitory peptide

derived from CaMKIIN, a 79-amino acid protein that binds activated CaMKII and potently inhibits its activity[213, 214]. The CaMKIIN peptide inhibited CaMKII binding to GST-NR2B and GST-β_{2a}, and did so more potently than did the NR2B peptide, AC-2 or His-β_{2a}(410-505)(**Figure A1**). There are two possible interpretations of this observation: either the unconserved residues in CaMKIIN interact with the same sites on CaMKII (i.e, by a similar mechanism) as the conserved residues in NR2B and β_{2a} or they engage different amino acids on CaMKII (i.e, bind by a different mechanism) but are still able to physically or sterically block CaMKII-access to other binding partners.

Ca_v β_{2a} inhibits CaMKII activity

The competition studies in the previous section indicated that β_{2a}, like NR2B and CaMKIIN, binds the catalytic site of CaMKII, and therefore suggested that β_{2a} would inhibit CaMKII. Indeed, β_{2a} inhibited CaMKII activity toward syntide-2, the model peptide substrate, which is consistent with β_{2a} binding at or close to the active site of CaMKII (**Figure A2**). GST-NR2B inhibited CaMKII activity more potently and completely than did GST-β_{2a}. Even though this interesting observation needs to be replicated to make any meaningful comments, it suggests NR2B binds tighter to CaMKII than β_{2a} under these conditions. However, earlier studies showed that NR2B and β_{2a} bind CaMKII with a similar apparent affinity (**Figure 7**). The discrepancy of the inhibition assay may result from multiple reasons, including differences in phosphorylation and outright experimental error. Furthermore, the in-solution nature of these studies could

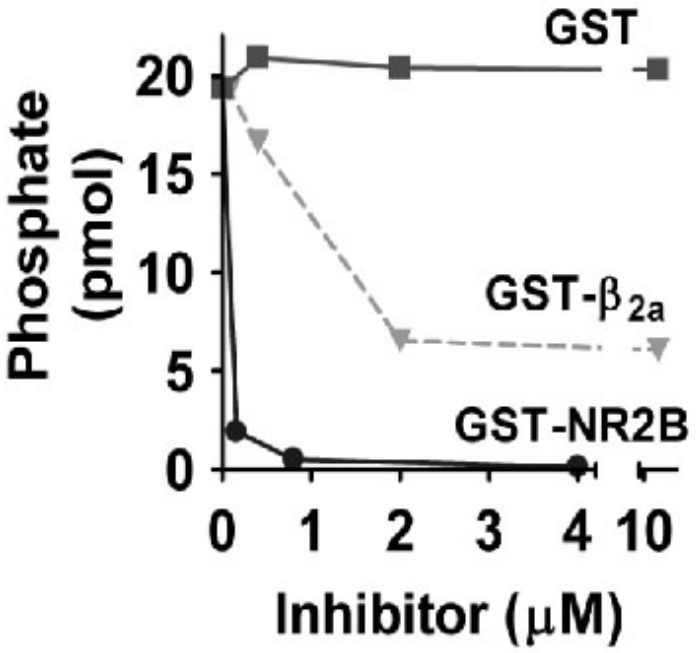

Figure A2. Inhibition of CaMKII activity by β_{2a}
Ca^{2+}/CaM-dependent activity of CaMKIIα (20 nM) was assayed with syntide-2 substrate (20 μM) in the presence of increasing concentrations of GST, GST-NR2B [1260-1339] or GST-β_{2a}[1-604]. Both proteins except GST potently inhibited CaMKII activity. *This experiment was performed by Dr. Alfred J. Robison (The author of this thesis failed to locate the original record of this interesting experiment but it is likely that the NR2B and GST-* β_{2a} *were wild type proteins, not their respective S1303A and Thr498A mutants).* .

reveal different affinities compared to the affinities determined in assays using immobilized proteins. Fluorescence anisotropy (**see chapter 4**) studies may resolve the potential differences in CaMKII-NR2B/β_{2a} affinities determined in the inhibition and binding assays.

Association of LTCCs and CaMKII in Brain Fractions

LTCC subunits exhibit differential subcellular localization, which may influence their association with CaMKII (**see Introduction**). To explore the association of CaMKII and LTCCs in various cellular compartments, I immunoprecipiated CaMKII from cytosolic, membrane enriched, Triton-soluble and deoxycholate-soluble (presumably particulate) fractions of mouse forebrain. The $Ca_v1.2\alpha_1$ was present in all but the cytosolic fraction, and particularly enriched in membrane fractions. Unlike $Ca_v1.2\alpha_1$, the β_1 and β4 subunits were detected in the cytosolic fraction, likely because they are soluble Ca_v subunits. Relative to the cytosolic fraction, the β subunits was enriched in other brain fractions, in parallel with $Ca_v1.2\alpha_1$ and CaMKII (**Figure A3**). Complexes of the $Ca_v1.2\alpha_1$ and β_1 subunits and CaMKII were also present in CaMKII immunoprecipitates from the non-cytosolic fractions. There was virtually no β_4 staining in the CaMKII immuniprecipates, except for a weak band in CaMKII complexes isolated from membrane-enriched fractions. Additional experiments are needed to verify the specificity of the β_4 band. In combination, these data demonstrate that CaMKIIα is present in the same brain fractions as LTCC subunits and is associated with a subset of LTCCs containing β_1 subunits.

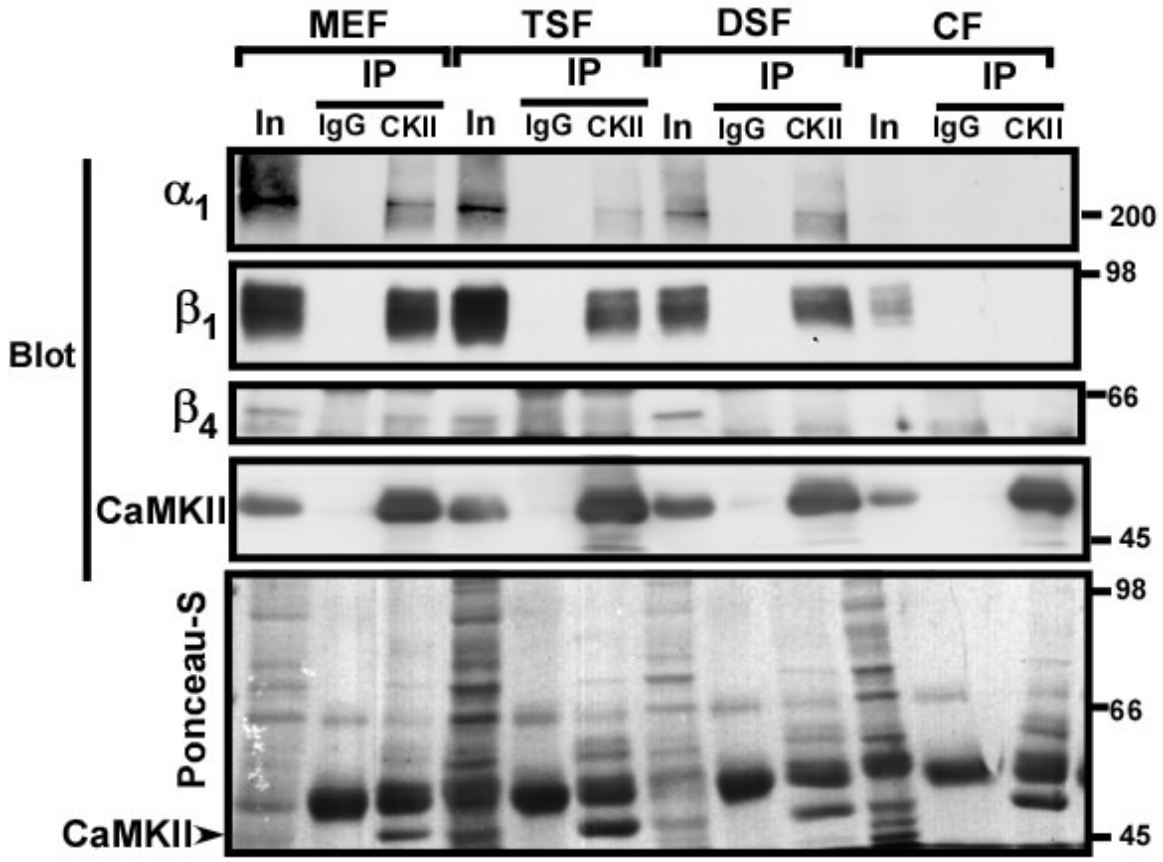

Figure A3. CaMKII association with LTCC subunits in brain fractions. CaMKII immune complexes isolated from subcellular fractions of mouse forebrain were western blotted using the $Ca_v1.2\alpha_1$, β_1 or β_4 subunit antibodies: Triton-soluble fraction (TSF), deoxycholate-soluble fraction (DSF), membrane enriched fraction (MEF) or cytosolic fraction (CF). The bottom panel shows Ponceau-S stained membranes of total protein loaded. n = 1

The β_{2a} subunit enhances CaMKII association with full-length $Ca_V1.2$ α_1

Although we showed CaMKII Co-IPs with β_{2a} under basal conditions (Grueter 2006), we did not test if under these conditions the β_{2a} subunit also targets CaMKII to full-length $Ca_v1.2\alpha_1$ subunits. I therefore transfected HEK293 cells to express EGFP-CaMKII in the absence or presence of HA-$Ca_v1.2\alpha_1$ subunits and WT or L493A mutated FLAG-β_{2a}. Unlike the previously described studies where transfected cells were pre-treated with Ca^{2+} ionophore to boost CaMKII activation (**Figure 22**), in these studies cells were not pre-treated. The HA-$Ca_v1.2\alpha_1$ immune complexes were western blotted for HA, FLAG and CaMKII. HA-$Ca_v1.2\alpha_1$ subunits were readily detected in HA-immune complexes and co-expression of β_{2a} (WT or L493A) enhanced the levels of α_1 subunit expression (**Figure A4**). CaMKIIα was weakly detected in immune complexes isolated from cells expressing only the $Ca_v1.2\alpha_1$ but the detection was enhanced following co-expression of FLAG-β_{2a} WT subunit. The L493A mutation virtually reversed the β_{2a}-dependent enhancement of CaMKII in LTCC complexes without affecting the amount of α_1 the subunit. Thus, the β subunits target CaMKII to the full-length $Ca_v1.2\alpha_1$ under these basal conditions.

The β_{2a} subunit enhances association of CaMKII<u>δ</u> with full-length $Ca_V1.2$ α_1

Several experiments in Chapter 2 used CaMKIIδ to test CaMKII binding to β_{2a} in binary complexes. To determine if β_{2a} enhances the assembly of CaMKIIδ isoform with LTCC complexes, LTCC subunits were transfected with MYC-CaMKIIδ_2(T287D). The constitutively active mutant was used to enhance CaMKII- β_{2a} interaction (please read the main document, especially **Chapter 2**, first!). CaMKIIδ_2(T287D) was detected in anti-HA immune complexes isolated from cells expressing WT FLAG-β_{2a} (**Figure A5**) but not in anti-HA immune complexes isolated from cells expressing L493A-mutated FLAG-β_{2a} or from cells

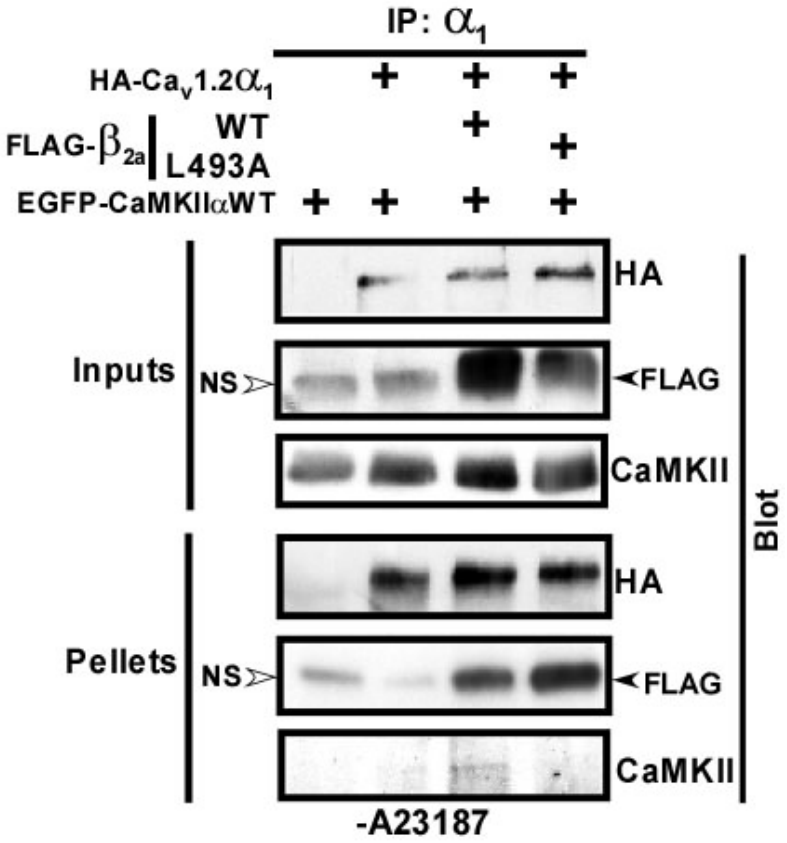

Figure A4. The β_{2a} subunit targets CaMKIIα to $Ca_v1.2$ α_1 subunits in heterologous cells.
HEK293 cells were transfected to co-express EGFP-CaMKIIα, HA-$Ca_v1.2$ α_1 and/or FLAG-β_{2a} (WT or L493A) as indicated. Triton-soluble fractions (Inputs) and $Cav1.2\alpha_1$ immune complexes (pellets) were western blotted for HA, FLAG, or EGFP. These data are representative of at least 3 similar experiments.

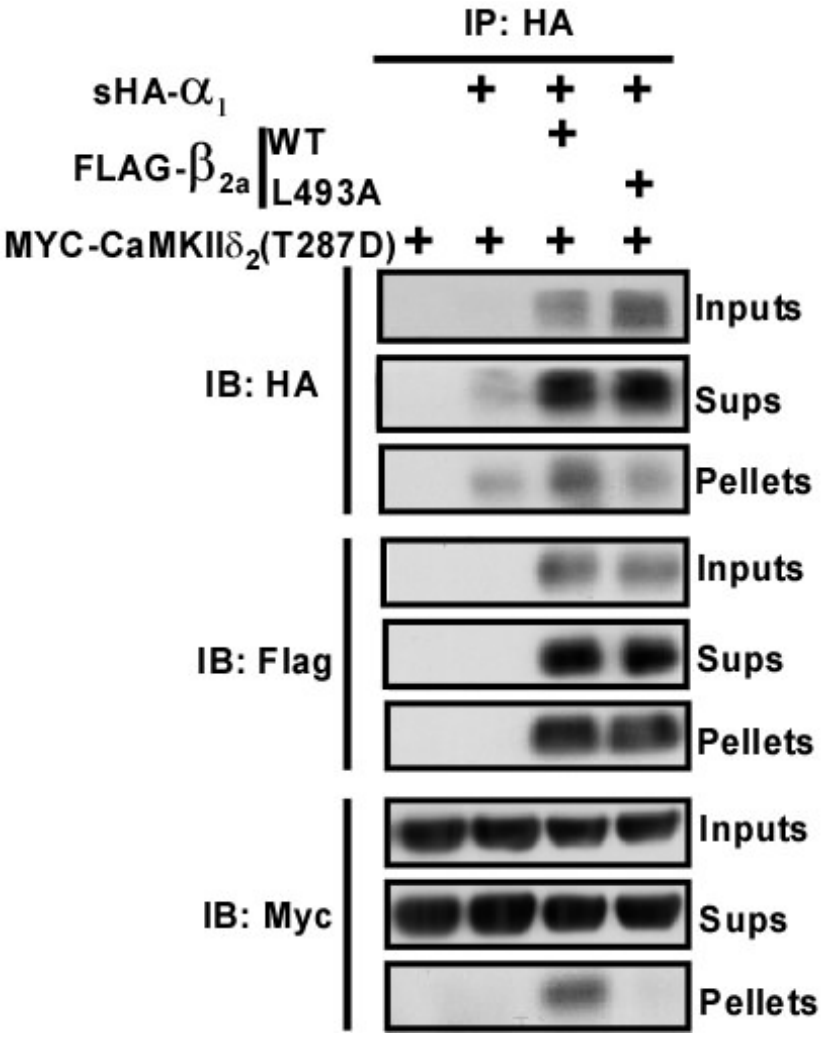

Figure A5. The β_{2a} subunit targets CaMKIIδ_2(T287D) to $Ca_v1.2$ α_1 subunits in intact HEK293 cells.
HEK293 cells co-expressing MYC-CaMKIIδ_2(T287D), HA-$Ca_v1.2$ α_1 and/or FLAG-β_{2a} (WT or L493A) as indicated. Triton-soluble fractions (Inputs), supernates and anti-HA immune complexes (pellets) were western blotted for HA, FLAG, or MYC. These data are representative of 2 independent experiments.

that did not express FLAG-β_{2a}. These data demonstrate that the high affinity CaMKII interaction with the β_{2a} subunit promotes efficient assembly of multiple isoforms of CaMKII with LTCCs.

The β_{2a} subunit enhances CaMKII colocalization with recombinant full-length α_1

Even though immunoprecipitations are useful to show physical association of proteins, they are not useful for localizing protein complexes in intact cells. Furthermore, they may misrepresent the true nature of protein complexes, for example due to artifacts introduced by protein dilution during lysis and wash procedures. Thus, I analyzed the colocalization of CaMKIIα and $Ca_v1.2\alpha_1$ in intact cells (**see supplemental methods at the end of this section**). The intensity correlation analysis (ICA) method of quantifying colocalization was first validated by showing that EGFP-CaMKIIα but not EGFP colocalized with HA-$Ca_v1.2$ after Ca^{2+} ionophore treatment (**Figure A6**). The ICA method was then used to assess the colocalization of mCherry-CaMKIIα and EGFP-LTCC complexes coexpressed without the β subunit, with HA-β_{2a} or HA-β_4 following ionophore treatment. All three proteins formed fluorescent puncta, though CaMKII and $Ca_v1.2\alpha_1$ puncta were not as prominent as the β subunit puncta. The HA-β_4 protein was also present in the nucleus, which is consistent with its previously reported nuclear localization[47, 215]. In the absence of HA-β_{2a} the ICQ of EGFP and mCherry was high, which is consistent with the β_{2a}-independent association of $Ca_v1.2\alpha_1$ and CaMKIIα under these conditions in Co-IPs (**Figure 22**). The HA-β_{2a} enhanced colocalization of mCherry-CaMKIIα with

EGFP-$Ca_v1.2\alpha_1$ by ~40% but the β_4 isoform that does not bind CaMKII (**Figure 10 and 11**) did not (**Figure A7**). Thus, under conditions that activate CaMKII, the β_{2a} but not the β_4 subunits enhanced CaMKII association of $Ca_v1.2$ in intact cells. Unactivated CaMKII does not bind the β subunit (**Figure 7 and 10**) and thus is not tethered to the I/II linker (**Figure 21**), suggesting that CaMKII and LTCCs should not colocalize under basal conditions, without Ca^{2+} ionophore treatment. Omission of Ca^{2+} ionophore prevented the enhancement of mCherry-CaMKIIα colocalization with EGFP-$Ca_v1.2\alpha_1$ by HA-β_{2a}(**Figure A8**). However, the lack of β_{2a}-dependent increase in CaMKII-LTCC colocalization under basal conditions appears to contradict the coimmunoprecipitation studies in **Figure A4** where CaMKII is targeted, albeit weakly, to $Ca_v1.2\alpha_1$ by the β_{2a} under basal conditions. The discrepancy may be due to differences in the sensitivity of immunofluorescence and western blotting to CaMKII. Some suggested followup studies include the use of β_{2a} (L493A), $Ca_v1.2\alpha_1$ (W470A), which should interfere with CaMKII targeting (please see main document) or varying ratios of $Ca_v1.2\alpha_1$, β_{2a} and CaMKII to get optimal ICQ changes.

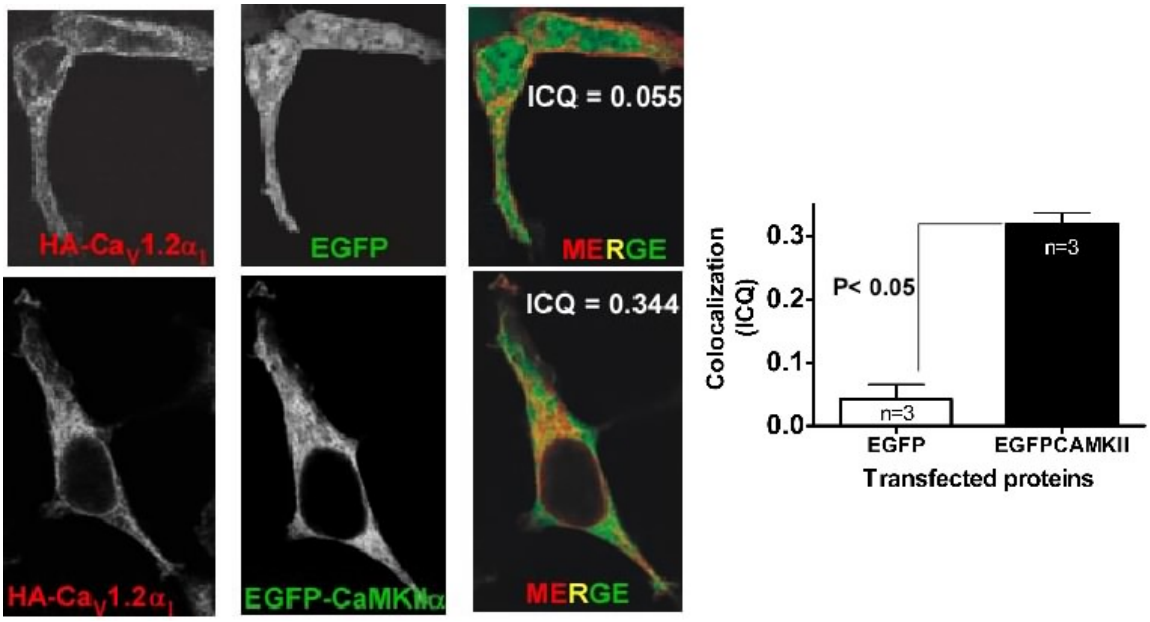

Figure A6. Validation of ICA method of Colocalization Analysis.
To validate the method HEK293 cells plated in 12-well plates were transfected to coexpress HA-$Ca_V1.2\alpha_1$ (5 µg DNA per well) and FLAG-β_{2a} (2 µg DNA per well) with EGFP or EGFP-CaMKII (1 µg DNA per well). They were then fixed, permeabilized, stained for HA and imaged by confocal microscopy. The ICQs were generated as described by Li et al and compared using a student's t-test (*p< 0.05, n = 3 cells).

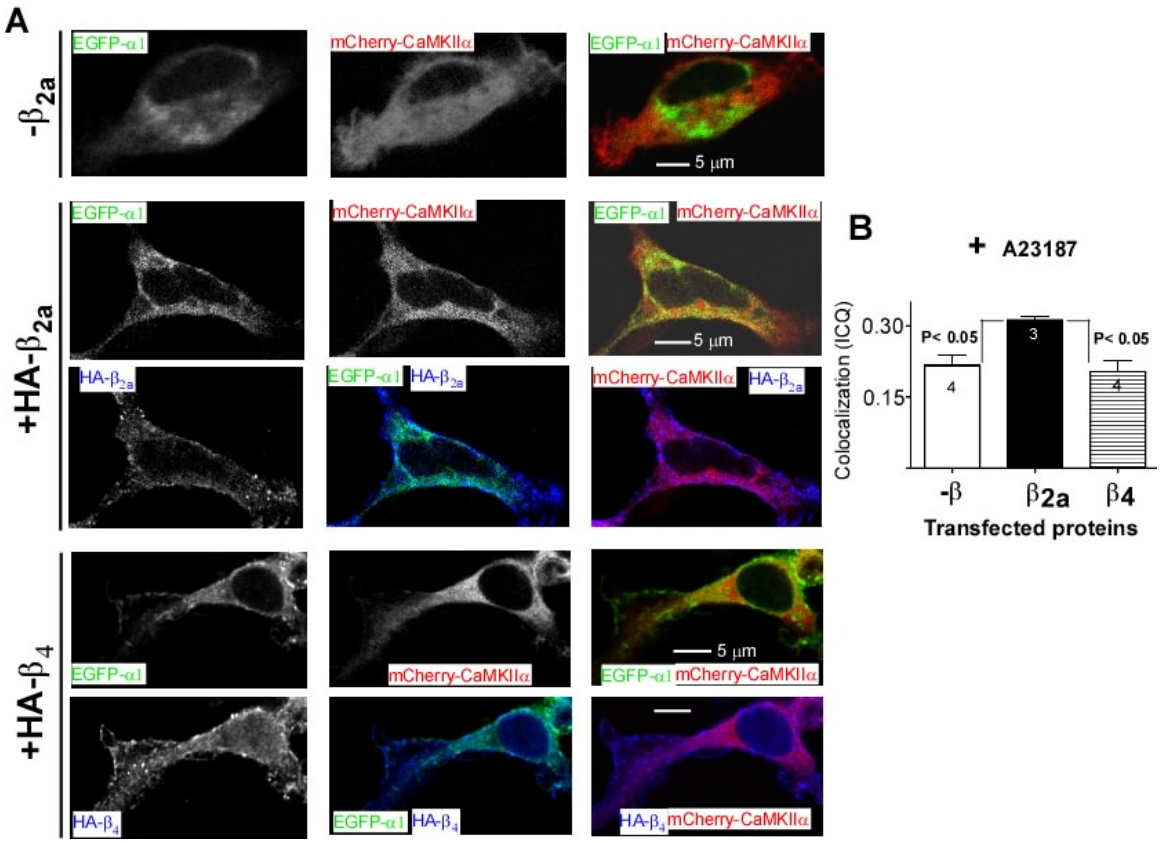

Figure A7 Differential targeting of CaMKII to full-length $Ca_v1.2\alpha_1$ subunit in by β_{2a} and β_4 in intact cells.

(**A**) HEK293 cells were transfected to coexpress EGFP-$Ca_V1.2\alpha_1$ (5 μg DNA per well) and mCherry-CaMKIIα(1 μg DNA per well) with HA-β_{2a} or HA-β_4 (2 μg DNA per well). After 36 hours they were treated with 10 μM A23187 for 5 minutes, fixed, permeabilized, stained for HA and imaged by confocal microscopy.

(**B**) Intensity correlation quotients (ICQ) of the images were generated as described at the end of the **Appendix Chapter**. Average ICQs of 5 to 10 cells were calculated from multiple independent experiments (indicated on the bars) and statistical comparison of data were performed with one-way ANOVA followed by Newman Kewl's Multiple Comparison Test.

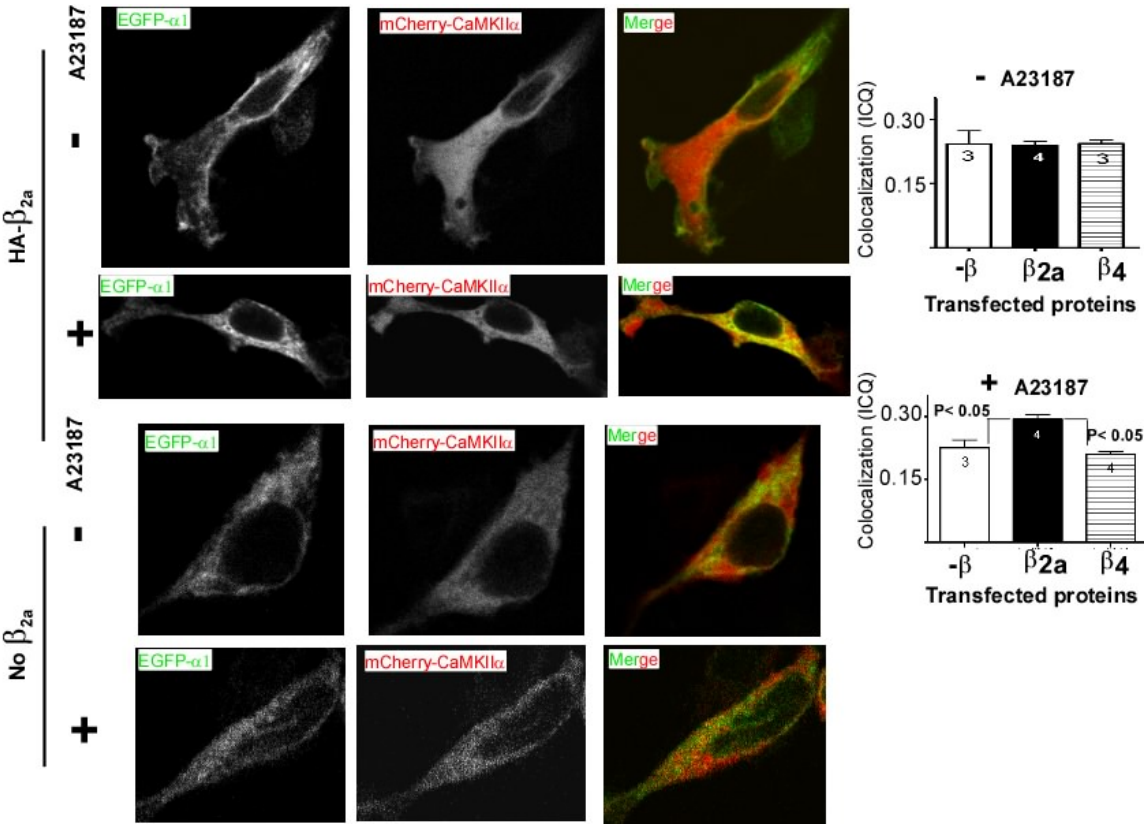

Figure A8. Activity-dependent targeting of CaMKII to full-length Cav1.2α$_1$ subunit in intact cells.

HEK293 cells were transfected to coexpress EGFP-Ca$_V$1.2α$_1$(5 µg DNA per well) and mCherry-CaMKIIα(1 µg DNA per well) with HA-β$_{2a}$ or HA-β$_4$ (2 µg DNA per well, images not shown). After 36 hours, they were treated with DMSO or 10 µM A23187 for 5 minutes, fixed, permeabilized, stained for HA and imaged by confocal microscopy. Intensity correlation quotients (ICQ) of the images were generated as described in at the end of this chapter. Statistical comparisons were performed as in **Figure A7**.

Ca_v β_{2a} subunit phosphorylation in binary β_{2a}-CaMKII complexes.

In **Chapter 3**, I explored the role of CaMKII binding in overall phosphorylation of $Ca_v1.2$ subunits and in specific phosphorylation of the β_{2a} subunit at Thr498 in LTCC complexes. However, these studies focused on the ternary α_1-β-CaMKII complexes, and did not establish if CaMKII binding controlled β_{2a} phosphorylation independently of the α_1 subunit. In a subset of the metabolic labeling studies in **Chapter 3**, I immunoprecipitated residual FLAG-β_{2a} from the supernatants of lysates that had previously been subjected to $Ca_V1.2\alpha_1$ immunoprecipitation. The enriched β_{2a} protein was visible by Ponceau-S stain but there was no comparable staining of α_1, suggesting the enriched β_{2a} pool is unlikely to be bound to α_1 (**Figure A9A**). ^{32}P incorporation into the apparently non-LTCC associated β_{2a} was unaffected by the L493A mutation. In additional studies, I determined that Thr498-specific phosphorylation of β_{2a} was also enhanced in lysates of cells cotransfected with only FLAG-β_{2a} and CaMKII and in binary FLAG immune complexes (**Figure A9B**). These preliminary studies imply that modulation of Thr498 phosphorylation is dependent on the CaMKII-β_{2a} interaction and qualitatively independent of any interaction of CaMKII with the associated α_1. (**Chapter 3** discusses this subject more exhaustively)

Loss of CaMKII binding to β_{2a} does not alter phosphorylation of β_{2a} at Thr498 within LTCC complexes in the presence of only endogenous CaMKII

Since HEK cells appear to express a low level of at least one endogenous CaMKII isoform[193, 194], I also examined the effect of CaMKII binding on β_{2a} phosphorylation at Th498 in the presence of only endogenous CaMKII. Mutation of β_{2a} to disrupt binding of CaMKII reduced phosphorylation of β_{2a} at Thr498 by 60% in total lysates but had no effect on Thr498 phosphorylation in

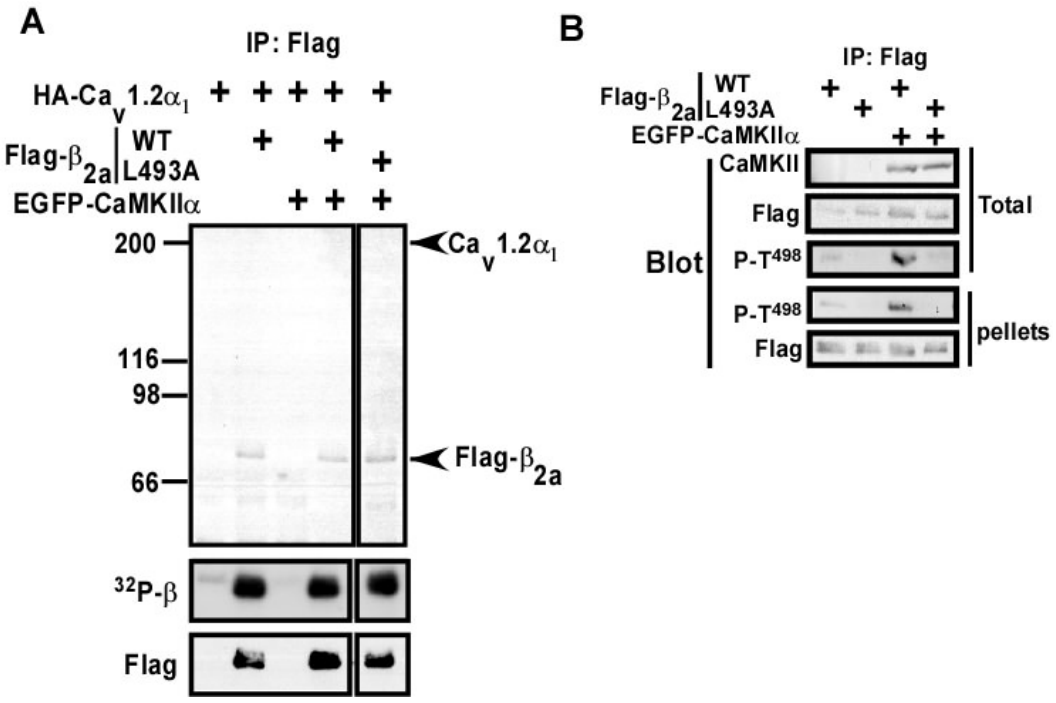

Figure A9. Phosphorylation of β_{2a} in binary complexes with CaMKII.
(**A**) HEK293 cells co-expressing HA-$Ca_v1.2$ α_1, EGFP-CaMKIIα and FLAG-β_{2a} (WT or L493A) were labeled with ^{32}P-phosphate (see Methods). β_{2a} was immunoprecipitated from supernates following the HA-$Ca_v1.2\alpha_1$ immunoprecipitation: Representative Ponceau-S stain of FLAG-β_{2a} showing enrichment of β_{2a} but not α_1 (α_1 normally migrates at the ~200 kDa as indicated), and ^{32}P-phosphorylation. *n = 4*

(**B**) Lysates of HEK293 cells co-expressing EGFP-CaMKIIα and FLAG-β_{2a} (WT or L493A), as indicated, were immunoprecipitated using anti-FLAG beads. The immune complexes were analyzed by western blotting as indicated. *n=2*

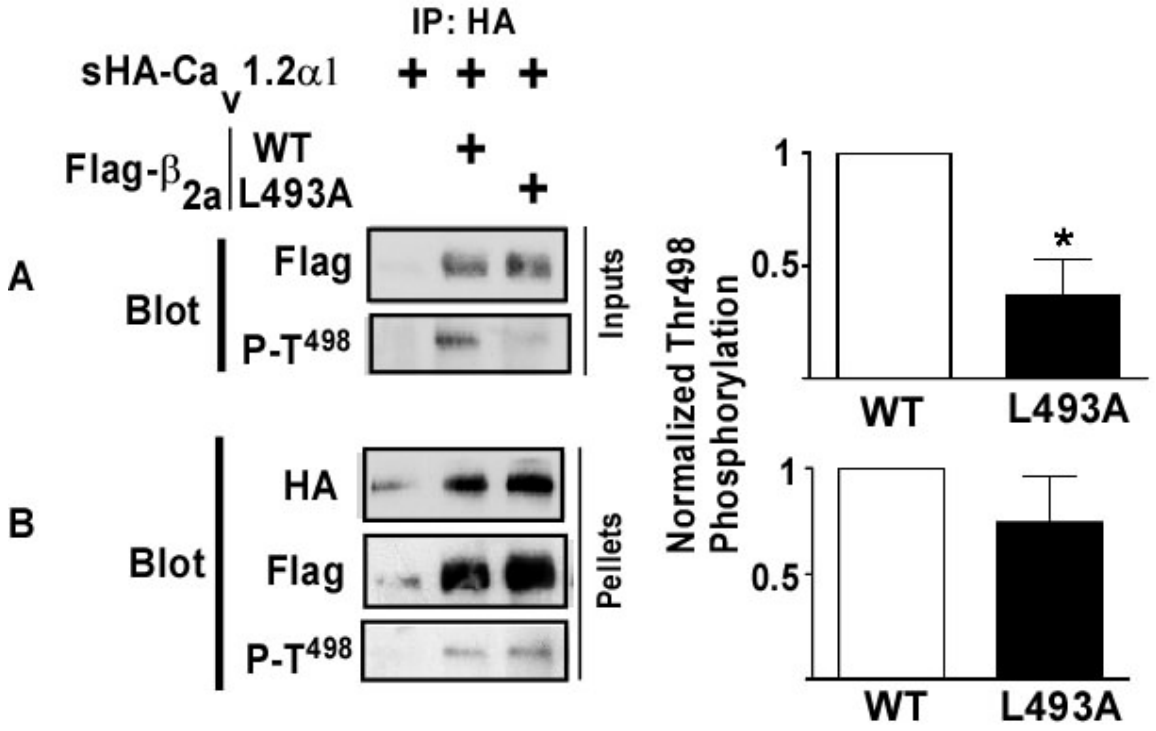

Figure A10. Binding of endogenous CaMKII enhances β_{2a} phosphorylation at Thr498.
Lysates of HEK293 cells co-expressing HA-$Ca_v1.2$ α_1 and FLAG-β_{2a} (WT or L493A), as indicated, were immunoprecipitated using anti-HA beads. Total lysates (**A**) and immune complexes (**B**) were analyzed by western blotting as indicated. Phosphorylation of wild-type and mutated FLAG-β_{2a} proteins at Thr498 was quantified. Phosphorylation of FLAG- β_{2a}(L493A) was normalized to that of wild-type β_{2a} and the mean±sem (n=3) is plotted: a one sample t-test was used for statistical comparison

immunoprecipitated LTCC complexes (**Figure A10**). These results suggest binding of the endogenous CaMKII isoform in HEK to LTCC is neither required nor sufficient to alter phosphorylation of Th498 within LTCC complexes, but is important in phosphorylating free β_{2a} at Thr498.

The effect of CaMKIIδ binding to β_{2a} on Thr498 phosphorylation

A variety of CaMKII isoforms are expressed in different tissues. The CaMKIIδ isoforms predominate in the heart and have been shown to facilitate LTCCs. I previously showed that the phosphorylation β_{2a} at Thr498 was unaffected by co-transfection CaMKIIδ_2 (**Figure 15A**), which contrasts with the effects of CaMKIIα binding and suggests effects of CaMKII isoform identity. To investigate in more detail the potential isoform selectivity in the effect of CaMKII-binding on Thr498 phosphorylation, I cotransfected HEK cells with MYC-CaMKIIδ_2 and FLAG-β_{2a}(WT or L493A) with or without the α_1 subunits. I first analyzed Thr498 phosphorylation in cells doubly transfected with MYC-CaMKIIδ and FLAG-β_{2a}. Mutation of Leu493 to Ala in β_{2a} reduced Thr498 phosphorylation in HEK293 *total lysates* containing MYC-CaMKIIδ_2 WT (**Figure A11**) but this effect was less severe than that in the presence of CaMKIIα (**Figure 26, 27 and A9**). Binding of MYC-CaMKIIδ_2 did not appear to alter Thr498 phosphorylation within immunoprecipitated FLAG-β_{2a}-CaMKII *binary complexes*. Thus, isoform identity appears to control the effect of CaMKII binding on phosphorylation.

I also compared Thr498 phosphorylation in the presence of CaMKIIδ_2 to that in presence CaMKIIδ_3. The CaMKIIδ_2 variant is primarily cytosolic but the CaMKIIδ_3 splice variant contains a nuclear localization sequence that can target CaMKIIIδ_3 to the nucleus[216]. Despite this difference, I observed little

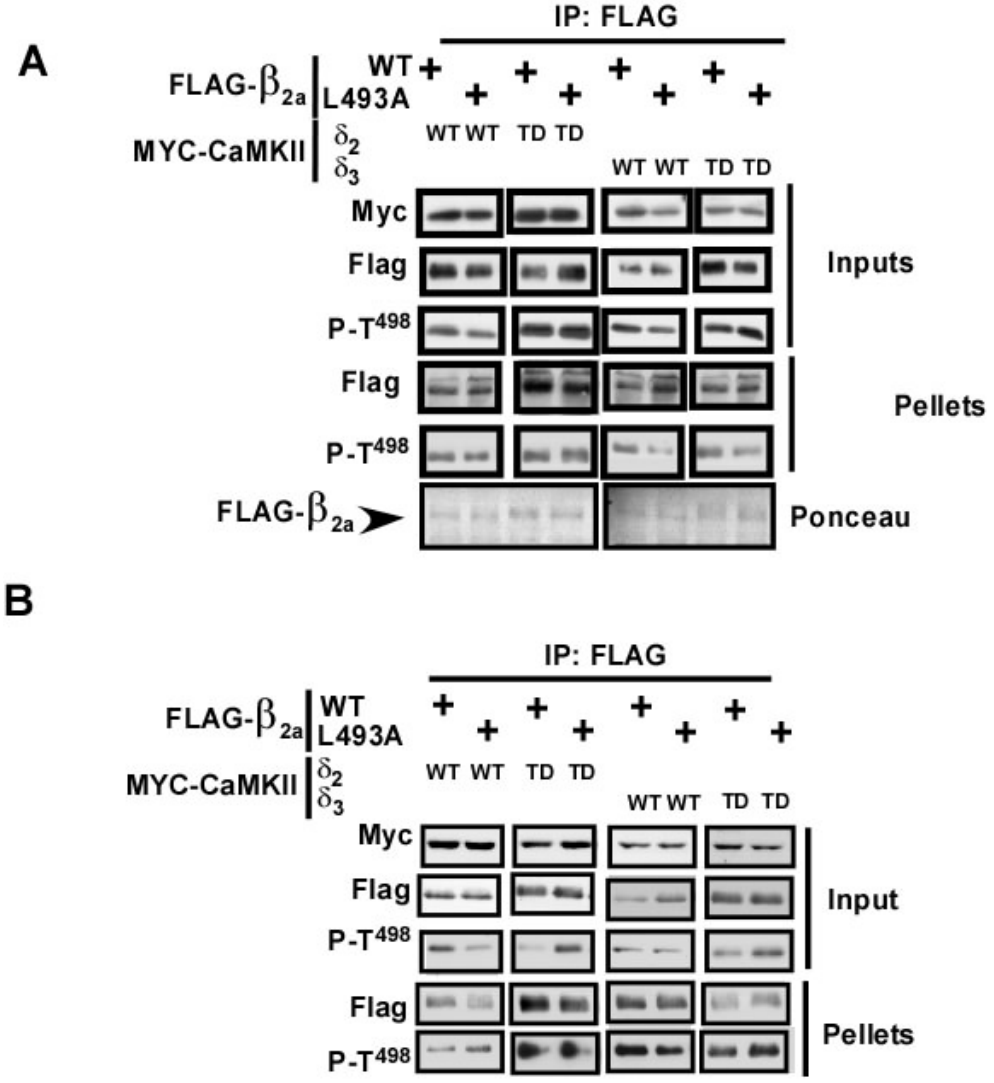

Figure A11. Effect of CaMKIIδ isoform binding on β_{2a} phosphorylation at Thr498 in Binary Complexes.
Lysates of HEK293 cells co-expressing FLAG-β_{2a} (WT or L493A) and MYC-CaMKIIδ_2 or δ_3 isoforms (WT or T287D) were immunoprecipitated using anti-HA beads. The immune complexes were analyzed by western blotting. **A** and **B** are replicate experiments.

difference between the two CaMKIIδ isoforms with regards to the extent of Thr498 phosphorylation or the effect of binding (**Figure A11**). Even though this would imply that the differential localization of CaMKII isoforms does not control Thr498 phosphorylation, it is possible that under my conditions the δ isoforms of CaMKII did not localize differentially within the cell. The actual localization of the isoforms was not determined in my studies.

Activation of CaMKIIα did not alter the effect of Leu493 mutation on Thr498 phosphorylation (**Figure 28**). I also tested if CaMKIIδ activation by mimicking Thr287 autophosphorylation (by T287D mutation) prevented the reduction in Thr498 phosphosphorylation by Leu493Ala mutation. MYC-CaMKIIδ_2 (T287D) or MYC-CaMKIIδ_3(T287D) both resulted in increased overall phosphorylation of $\beta_{2a,}$ as evidenced by a reduction in electrophoretic mobility of the immunoprecipitated FLAG-β_{2a} (**Figure A11**). When normalized to the total amount of $\beta_{2a,}$ phosphorylation of Thr498 did not appear to be enhanced by the CaMKIIδ(T287D) mutants as compared to WT CaMKIIδ. Even though these experiments are preliminary and the results inconsistent, the use of CaMKIIδ instead of CaMKIIα virtually overcame the Thr498 phosphorylation deficit caused by Leu493 Ala mutation (**Figure A11**), which confirmed my previous published observations (**Figure 15A**).

In addition to the above studes of binary β_{2a}-CaMKIIδ complexes, I also explored Thr498 phosphorylation in ternary α_1-β_{2a}-CaMKIIδ complexes. I cotransfected HEK293 cells with $Ca_v1.2\alpha_1$, MYC-CaMKIIδ_2 (T287D) or MYC-CaMKIIδ_3(T287D) and FLAG-β_{2a} (WT or L493A). CaMKII binding appeared to reduce Thr498 phosphorylation in total lysates or ternary LTCC complexes (**Figure A12**). However, it is important to note the differences in $Ca_v1.2\alpha_1$ subunit expression.

Thus, my studies with the CaMKIIδ isoforms implicate additional, isoform-dependent factors in controlling the effect of CaMKII binding on Thr498 phosphorylation. Whether the differences between CaMKIIα and CaMKIIδ isoforms are due to differences in their expression or activation remains inconclusive.

Overall, multiple factors would be predicted to determine the extent to which CaMKII binding affects Thr498 phosphorylation within β_{2a} in intact cells, including the activity, subcellular localization and isoform of CaMKII. Of these, the identity of the CaMKII isoform affected the binding-dependent changes in Thr498 phosphorylation in my preliminary studies. The various factors remain to be more precisely defined.

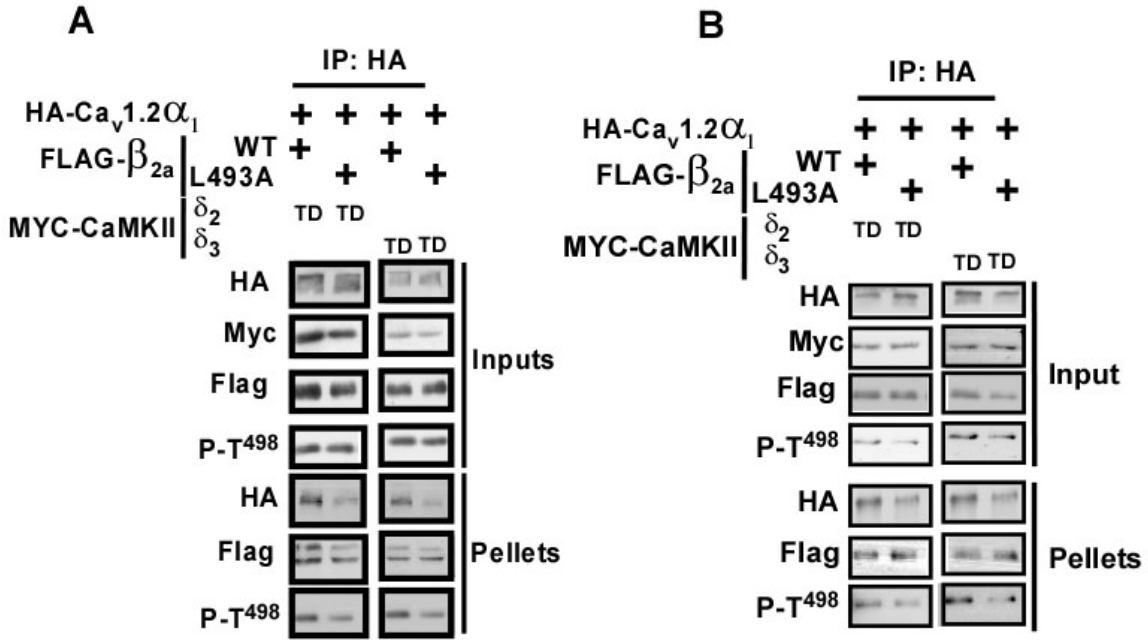

Figure A12. Effect of CaMKIIδ isoform binding on β_{2a} phosphorylation at Thr498 in Ternary Complexes.
Lysates of HEK293 cells co-expressing HA-Ca$_v$1.2α1, FLAG-β_{2a} (WT or L493A) and MYC-CaMKIIδ_B or δ_C isoforms (WT or T287D) were immunoprecipitated using anti-HA beads. The immune complexes were analyzed by western blotting. **A** and **B** are replicate experiments

Thr498 phosphorylation inhibits anchoring of CaMKII to $Ca_V1.2\alpha_1$ by β_{2a}

Phosphorylation of the β_{2a} subunit at Thr498 is critical for CaMKII-dependent facilitation of LTCCs in cardiomyocytes [79]. In addition, Thr498 phosphorylation interferes with CaMKII binding to the β_{2a} subunit *in vitro* (**Chapter 2**). I therefore, investigated whether phosphorylation of β_{2a} modulated CaMKII tethering to $Ca_V1.2\alpha_1$ subunits. In initial experiments, wild-type and Thr498Ala mutated His-β_{2a} subunits were phosphorylated with CaMKII. Immunoblotting using a phospho-specific antibody confirmed that Thr498 was phosphorylated in the wild type β_{2a}, but not in the T498A mutant (**Figure A13A**). The phosphorylated β_{2a} proteins were also used to tether CaMKII to the α_1 I/II linker in a glutathione-agarose cosedimentation assay. Prephosphorylation reduced CaMKII tethering by wild type but not T498A β subunit (**Figure A13B**). Next, I explored if phosphorylation of β_{2a} in cells alters targeting of CaMKII to the full-length channel.

Mimicking of β_{2a} Thr498 phosphorylation impairs targeting of CaMKII to full-length $Ca_V1.2$ α_1

The β_{2a} subunit is phosphorylated at Thr498 in transfected cells. Because of the difficulty in controlling the level of phosphorylation of β_{2a} and CaMKII in cells, I explored the role of Thr498 in controlling CaMKII targeting to $Ca_V1.2$ α_1 subunits by using mutated β_{2a} subunits. HEK293 cells were transfected to co-express CaMKIIα or CaMKIIδ_2(T287D) and HA-$Ca_V1.2$ α_1 subunit with β_{2a} subunits mutated to either prevent (Thr498Ala) or mimic (Thr498Glu) the effects of Thr498

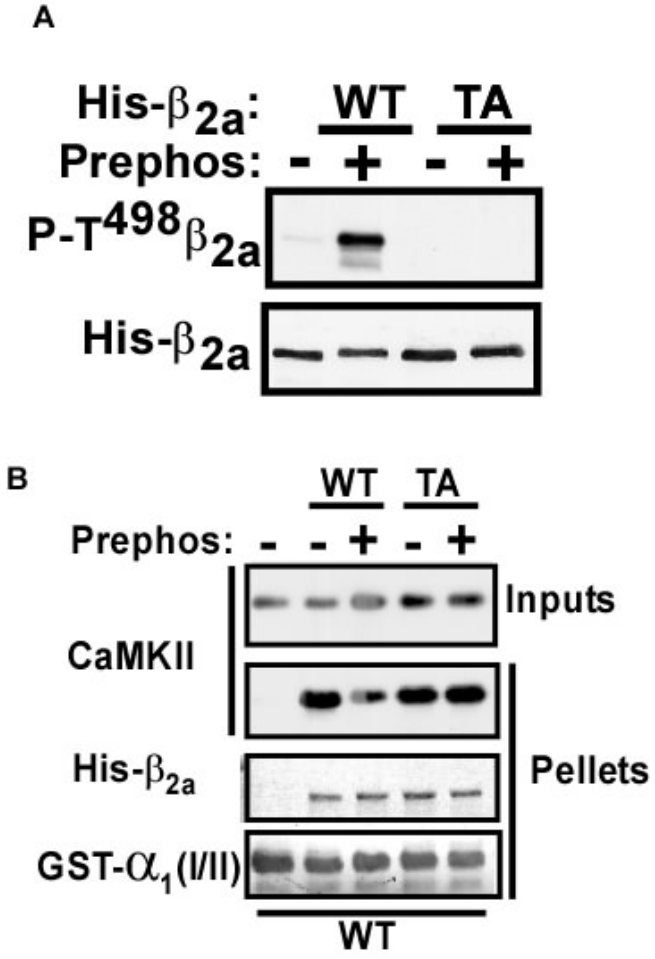

Figure A13. Anchoring of CaMKII to the α_1 by the β subunit is regulated by phosphorylation at Thr498.
His-β_{2a} (WT or T498A) was incubated at 37°C for 20 minutes with CaMKII, Ca^{2+}/CaM, in the presence (+) or in the absence (-) of ATP. The reaction was stopped by adding excess EDTA.

(**A**) T498 phosphorylation was demonstrated using an antibody that detects phospho-T498.

(**B**) Thr286-autophosphorylated CaMKIIα (50 pmol) was incubated with GST-Cav1.2α_1 I/II linker (WT) with and without His-tagged β_{2a} (WT or T498A). CaMKII cosediments with GST-Ca_v1.2 I/II linker only in the presence His-β_{2a}. Phosphorylation of β_{2a} at Thr498 reduces CaMKII anchoring by the β subunit. (n=3)

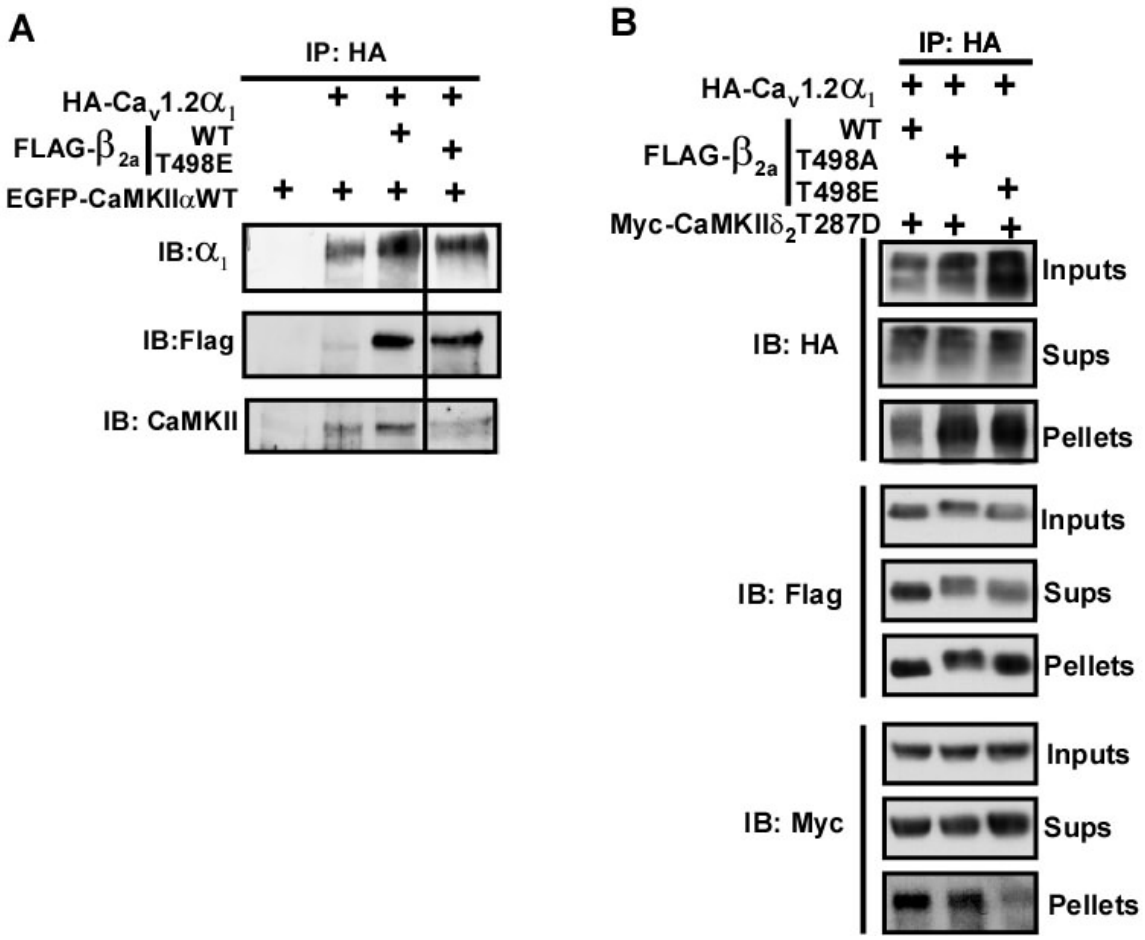

Figure A14 . Targeting of CaMKII to full-length α_1 subunit is regulated by Thr498 phosphorylation.

(**A**) HA-$Ca_v1.2$ was cotransfected into HEK293 cells with EGFP-CaMKIIα and Flag-β_{2a} (WT, T498A or T498E). $Ca_v1.2\alpha_1$ was then immunoprecipitated from cell lysates using an antibody against the HA tag. Immunocomplexes were analysed by western blotting for HA, FLAG, CaMKII. n =1

(B) myc-CaMKIIδ_2(T287D) targeting by β_{2a} (WT) and β_{2a} (T498E) to α_1 was compared as in (**A**). n = 1

phosphorylation. LTCC complexes were isolated by immunoprecipitation using antibodies to the HA-tag and then probed for the presence of β_{2a} and CaMKII. As repeatedly observed and mentioned through out this document (**please see Introduction, Chapter 3, Chapter 4, Figure 22, Figure A4, Figure A7, Figure A8**), there is some weak β subunit-independent association of CaMKIIα and $Ca_v1.2\alpha_1$. In contrast, CaMKIIδ does not appear to exhibit such β subunit-independent association with $Ca_v1.2\alpha_1$ (**Figure A5**). However, the reader should resist the temptation of meaningless speculation on this difference till CaMKIIα and CaMKIIδ targeting are compared in parallel experiments using the same antibody to probe for CaMKII. Compared to WT and T498A, the T498E mutation severely diminished CaMKII targeting to the α_1 by the β subunit (**Figure A14**). Based on these observations, it can be inferred that phosphorylation of Thr498 prevents CaMKII association with the LTCC complex. Furthermore, β_{2a} phosphorylation in preformed LTCC-CaMKII complexes is likely to dissociate CaMKII.

Time course of β_{2a} phosphorylation in LTCC complexes

As LTCC facilitation in cardiomyocytes occurs on a time scale of milliseconds to minutes, a process underlying facilitation such as phosphorylation would be expected to kick in before or during the timescale of facilitation. I therefore determined the time course of β_{2a} phosphorylation by associated CaMKII within the preformed complex on glutathione agarose beads. Addition of ATP to the complex resulted in a rapid reduction of the electrophoretic mobility of β_{2a}(WT)

within the first minute, consistent with the known multi-site phosphorylation, but the electrophoretic mobility of β_{2a}(T498A) was essentially unaltered even after a 20 minute incubation (**Figure A15A**). Notably, the GST-I/II linker band was not shifted on these gels, suggesting that it is not phosphorylated under these conditions. These observations are consistent with previous findings [150] that Thr498 to Ala mutation prevents phosphorylation at Thr498 and additional sites in β_{2a}. Thus phosphorylation of Thr498 within LTCC complexes is rapid, consistent with a role in facilitation.

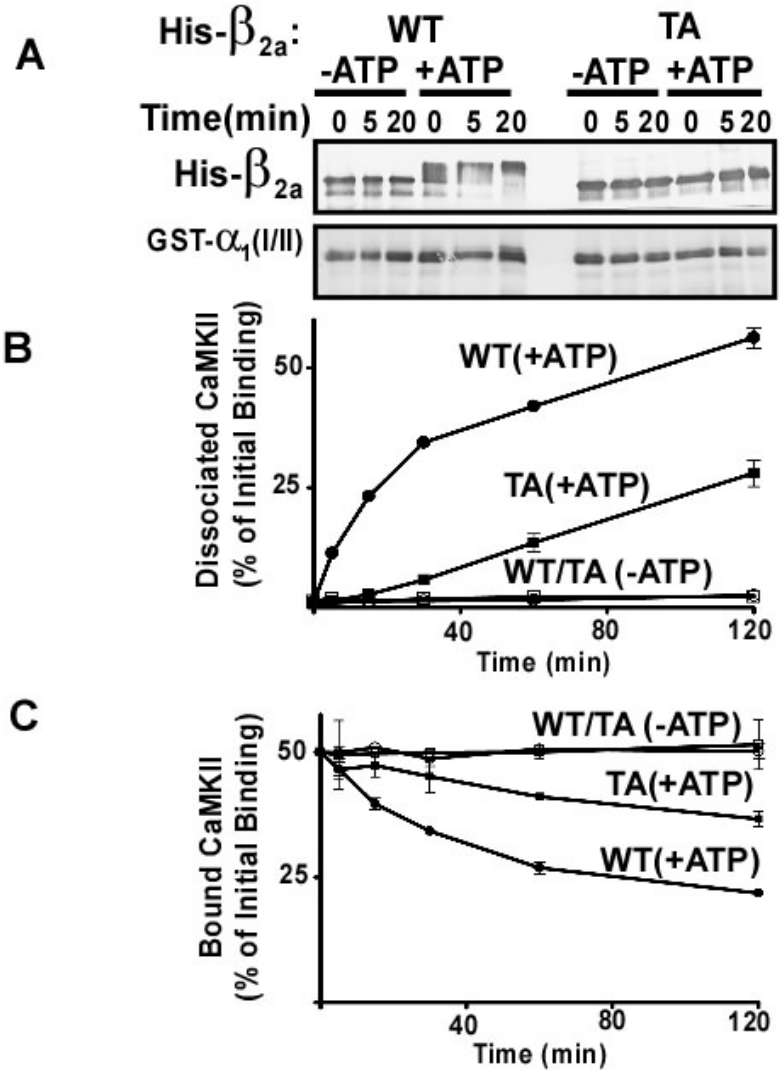

Figure A15. Phosphorylation of Thr498 dissociates CaMKII from LTCC complex.

(**A**) A preassembled complex of GST-I/II linker, His-β_{2a} (WT or T498A) and ^{32}P-labelled CaMKIIα was incubated with or without 0.5mM ATP. Aliquots of resin were removed at the indicated times and cosedimented proteins were analyzed by western blotting for His and GST.

(**B** and **C**) GST-α_1, β_{2a} and CaMKII complexes were preassembled in glutathione-coated wells and dissociation was initiated by addition of buffer or ATP. Aliquots of buffer were removed at the indicated times and CaMKII in the buffer (**B**) or remaining in wells (**C**) was quantified by scintillation counting. (n = 3, in some cases the error bars are smaller than the symbols).

Thr498 phosphorylation dissociates CaMKII from LTCC complexes *in vitro*

The effect of β_{2a} phosphorylation on CaMKII binding to GST-I/II was assessed using glutathione-coated plate binding assays, in which ^{32}P-labeled autophosphorylated CaMKII was tethered to the GST-I/II linker via either β_{2a}(WT) or β_{2a}(T498A). In the absence of added ATP, the tethering of CaMKII to GST-I/II by either form of β_{2a} was remarkably stable over a 2-hour time course. Addition of ATP to β_{2a}(WT) complexes resulted in time-dependent release of ≈50% of bound kinase over the 120 min time course, as assessed by the appearance soluble radioactivity (**Figure A15 B**) or a reduction in radioactivity remaining bound in wells (**Figure A15C**). Only ≈25% of the bound kinase was released after 15 minutes, even though the immobilized β_{2a} subunit appears to be maximally phosphorylated at this time (**Figure A15A**). Notably, addition of ATP induced only a slow dissociation of CaMKII from β_{2a}(T498A) complexes, with no significant dissociation after 15 minutes and only ≈25% dissociation after 2 hours. These data suggest that phosphorylation of β_{2a} at Thr498 plays a key role in destabilizing the tethering of CaMKII to the I/II linker domain of the $Ca_V1.2\alpha_1$ subunit. However, it appears that phosphorylation of β_{2a} at other sites or nucleotide binding also contribute to the dissociation of CaMKII. However, CaMKII dissociation in vitro occurs on a much slower time-scale than facilitation. It will be interesting to determine how dissociation correlates with recovery from facilitation.

Supplemental immunocytochemical methods

Immunocytochemistry: HEK293 cells were plated on poly-D-lysine-coated 18 mm coverslips (Fisher Scientific, Warrington PA) and transfected to coexpress EGFP-$Ca_V1.2\alpha_1$ and mCherry-CaMKIIα with HA-β_{2a} or HA-β_4. After 36 hours, A23187 (10 μM) was added and the cells were incubated for 5 minutes at 37^{o}C to boost CaMKII activation. The cells were fixed in 4% paraformaldehyde, 4% sucrose in phosphate buffered saline (PBS) for 20 minutes at 37^{o}C and permeabilized at room temperature for 5 minutes with 0.1% Triton-X 100 diluted in PBS. After a 1-hour blocking with 10% Donkey serum, the cells were incubated overnight with anti-HA (Covance, Berkeley, CA) and the following day with Alexa-Fluo 647 (Molecular Probes, Eugene, OR) secondary antibody. After rinsing, the coverslips were mounted onto slides (Fisher Scientific, Pittsburgh, PA) using Aqua Polymount solution (Polysciences, Warrington, PA). They were then imaged using a Zeiss LSM 510 inverted confocal laser scanning microscope with a Plan-Apochromat 63X oil immersion objective (NA 1.4) available at the Cell Imaging Shared Resource Core at Vanderbilt. The excitation wavelength/emission filter combinations used were: 488 nm / 505-550 nm for EGFP; 543 nm/ 560-615 nm for mCherry and 633 nm/long-pass 650 nm for Alexa-Fluor 647.

Intensity correlation analysis (ICA): ICA is a method that compares pixel intensities in two images and derives a numerical Intensity Correlation Quotient (ICQ) that determines colocalization (ICQ >0) random assortment (ICQ =0) or

segregation (ICQ<0) of the pixels[217]. To validate the method, HEK293 cells were transfected to coexpress HA-$Ca_V1.2\alpha_1$ and Flag-β_{2a} with EGFP or EGFP-CaMKII. They were then fixed, permeabilized, stained for HA and imaged by confocal microscopy. The ICQs were generated as described in reference [217](**Figure A6**).