CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II REGULATES CARDIAC L-TYPE CA^{2+} CHANNELS VIA THE BETA SUBUNIT

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CHAPTER I

INTRODUCTION

The functions of the heart have intrigued scientists for over 2000 years. In ancient times, the heart was thought of as the body's soul and even today is associated with human emotion. However, since 1628 when William Harvey described the circulatory system we have come to understand that the heart is an intricate biological pump that beats without pause for life (Bodi et al., 2005). It is estimated that the human heart beats over two billion times. Each beat is a result of the integration of ionic current resulting in mechanical output, both of which must be transmitted precisely in time and space. In 1883 Sydney Ringer discovered that calcium (Ca^{2+}) is a critical mediator of this process later termed excitation-contraction coupling (Ringer, 1883).

Heart disease is the number one cause of death in the United States (Thom et al., 2006). There are many forms of heart disease including heart failure and arrhythmias. One underlying theme in heart disease and many other diseases is disrupted Ca^{2+} homeostasis. Calcium is a charge carrier and universal mediator of diverse cellular processes. In cardiac myocytes, these processes include excitation-contraction coupling, gene transcription and apoptosis. Thus, intracellular Ca^{2+} operates core functions ranging from contraction that is required for all vertebrate life, to programmed cell death. Ca^{2+} enters cardiac myocytes through L-type Ca^{2+} channels (LTCC) where it activates

signaling molecules such as the multifunctional Ca^{2+}/calmodulin dependent protein kinase II (CaMKII). CaMKII is one of many specialized proteins poised to respond to Ca^{2+} signaling in cardiac myocytes. Growing evidence has linked CaMKII signaling events to normal and pathological conditions in the heart.

An overview of cardiac physiology

Gross cardiac anatomy

The heart is a muscle; its function is to contract, creating a pressure gradient to pump blood throughout the circulatory system. Mammals have a four-chambered heart consisting of two atria and two ventricles (Figure 1). The right atria collects oxygen depleted blood from the systemic circulatory system and transfers it to the right ventricle. The right ventricle contracts sending blood through the pulmonary arteries to the lungs where the blood becomes oxygenated. The oxygen rich blood returns to the left atria and is transferred to the left ventricle. The left ventricle comprises a majority of the mass of the heart. Upon contraction, it is responsible for pumping blood throughout the organs of the body.

After centuries of study, the organization of myoctyes within the ventricles still remains controversial (Anderson et al., 2005; Woodcock and Matkovich, 2005). The heart as a muscle is distinct from skeletal muscle in that it has no origin or insertion. It is formed from blood vessels during development. The architecture of the left ventricle wall consists of three layers. From outside to

inside are the epicardium, myocardium and endocardium (Anderson et al., 2005; Woodcock and Matkovich, 2005). It is accepted that the layers of cells within the ventricles are arranged in a pattern that enables the left ventricle to contract in a twisting manner. The epicardium and myocardium are oriented at 90° relative to one another and at 45° relative to the equatorial axis. The cells within the myocardium are oriented parallel to the equatorial axis of the heart (Anderson et al., 2005; Woodcock and Matkovich, 2005).

Structure of a myocyte

Cardiac myocytes are large multinucleated cells that are rich in mitochondria. They are highly structured rectangular cells that are aligned in an orderly fashion to function as one contractile unit. Cardiac myocytes directly interact with each other at each end of the cell and at various points along the long axis. The sites of cell-cell contact form intercalated discs. These discs are specialized structures that directly connect the cells and contain gap junctions, allowing passage of ions and peptides between cardiac myocytes. They also function to enhance electrical conduction throughout the ventricle. Further details about electrical conduction are discussed below (Ganong, 1999; Woodcock and Matkovich, 2005).

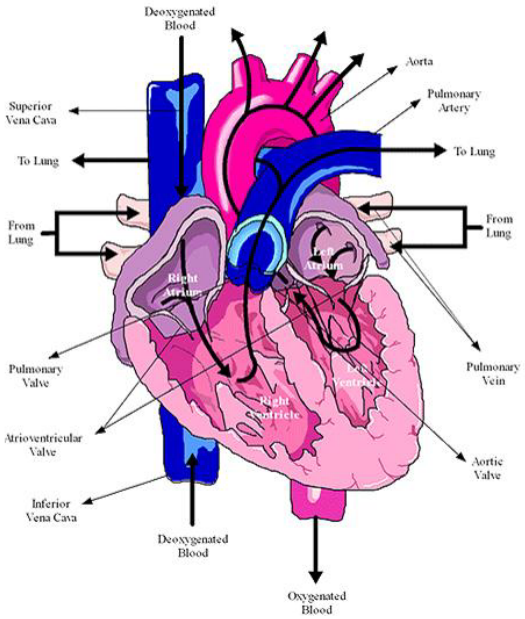


Figure 1. Anatomy of the heart and blood flow
The diagram represents a cross section of the heart labeled with: the four chambers: right atrium, right ventricle, left atrium, left ventricle, valves and major blood vessels. The arrows depict the flow of blood entering the right atria from the vena cava, passing through the atrioventricular valve into the right ventricle. From the right ventricle deoxygenated blood flows through the pulmonary artery to the lungs. Blood becomes oxygenated and enters the left atrium from the pulmonary vein. The blood flows through the left atrial ventricular valve into the left ventricle. The left ventricle contracts, pumping blood through the aorta and the entire body. (Modified from Wikipedia, September 2006)

Transverse tubules

Adult mammalian cardiac myocytes have adapted structurally to convert an electrical impulse into a functional output in a timely manner. Cardiac myocytes contain an elegant transverse-tubular (T-tubules) system that facilitates the process of E-C coupling. The T-tubules are structural components that function to position proteins involved in Ca^{2+} cycling. Among the proteins positioned within the T-tubules are the Na^{+}/Ca^{2+} exchanger, Na^{+} channels and the LTCCs (Mohler et al., 2002). Directly opposed to the LTCC on the sarcoplasmic reticulum (SR) are ryanodine receptors (RyR). RyRs are large Ca^{2+} channels that function to regulate Ca^{2+} efflux from the SR. The SR Ca^{2+} release constitutes a striking majority of the increase in intracellular Ca^{2+} concentration. Ca^{2+} within the SR is buffered by calsequestrin and is thought to be positioned in a manner to enhance the efficiency of Ca^{2+} efflux (Knollmann et al., 2006). Upon coordinated Ca^{2+} release via RyR the intracellular Ca^{2+} concentration reaches a threshold for activation of the contractile machinery (Song et al., 2005).

Contractile machinery

The function of a myocyte is to contract. The contractile machinery of a cardiac myocyte consists of a Ca^{2+} sensor, Troponin C, a structural component, actin, and the enzyme myosin. When the Ca^{2+} concentration increases to a sufficient level to bind to troponin C, this causes a conformational change in troponin C revealing an actin-binding site. Binding of troponin C to actin leads to

a direct interaction of actin with myosin. ATP bound to myosin is hydrolyzed and the power stroke by myosin leads to sliding of actin on myosin. The sarcomere is shortened by about 10nm per power stroke. The cumulative effect is cardiac myocyte shortening by about 10% of its diastolic length. The process is reversed upon a decrease in intracellular Ca^{2+} concentration by Ca^{2+} uptake into the SR (Ganong, 1999).

Electrical conduction in the heart

Action potential (AP) propagation in the myocardium occurs via electrical conduction from cell to cell through gap junctions. The firing of an AP in specialized cells is conducted by functional syncytia throughout the atria or ventricles. The specialized myocytes involved in pacemaking are the Sino-Atrial node (SA node). Other self-depolarizing cardiac myocytes include cells in the Atrial-Ventricular node (AV node) and Purkinje fibers. The pace of the heart rate is determined by the fastest of these cells that are typically those in the SA node (Figure 2). The AP then spreads throughout the ventricle via cell-cell contact to act as one functional unit.

Precise timing of cardiac electrical conduction is necessary for cardiac contraction and therefore blood flow. Alterations in the conduction lead to disease states, specifically arrhythmias. As mentioned previously, regulation of the heart rate is mainly a function of the SA node. The sympathetic nervous system can regulate the heart rate by direct innervation and hormonal release (epinephrine). Parasympathetic neural activity decreases heart rate via

acetylcholine (Ganong, 1999). Together these regulatory mechanisms establish the heart rate at rest and under stress.

Cardiac disease

The critical need for mechanical and therefore electrical spatio-temporal uniformity is apparent in most if not all forms of heart disease including cardiac arrhythmias, hypertrophy, and heart failure. Clinical treatment of these disease states has been challenging due to many factors including unknown causes of disease as well as adverse side effects of the current treatments. Targeting cardiac arrhythmias is extremely challenging mainly because the underlying mechanism for arrhythmias is unknown although many factors including LTCC and CaMKII misregulation have been implicated. Three separate mechanisms are thought to cause ventricular arrhythmias: re-entry, abnormal automaticity and triggered activity due to early-afterdepolarizations and delayed-afterdepolarizations (Winslow et al., 2005). Re-entry involves the depolarization of a region of the heart due primarily to alterations in the conductance pathway or conduction block. Abnormal automaticity occurs when cells within the heart other than the SA node are initiating the AP. Early-afterdepolarizations and delayed-afterdepolarizations are action potentials that are membrane depolarizations occurring before the cell completely repolarizes or in between normal APs. The treatment approaches taken are nonpharmacological (pacemakers, ablations etc) or pharmacological, although the options are limited and have not been proven to enhance mortality (Gilmour and Zipes, 2004). For ventricular arrhythmias,

common therapeutic treatments include Na^+ and K^+ channel blockers. These treatments are aimed at reducing the development of ventricular tachyarrhythmia's. Acceleration of I_{Ca} inactivation may be an important treatment strategy. Targeting arrhythmias requires more specific drug targets.

Heart failure is simply a condition in which the heart can't pump enough blood to properly supply the body. As the blood flow from the body to the heart backs up there is increased edema and overall congestion. Treatment is typically aimed at the volume overload involved with chronic heart failure and therefore diuretics are used. The use of angiotensin-converting enzyme (ACE) inhibitors which target angiotensin receptors, β blockers and aldosterone blockers are typical therapeutic approaches to treating congestive heart failure (Landmesser and Drexler, 2005). The role of CaMKII in different cardiac disease states is addressed in later sections of this chapter.

The Cardiac Action Potential

The cardiac AP varies slightly within the heart depending on the cell type. The separation of charge by the plasma membrane and the balance between intra- and extracellular ionic concentrations establish the membrane potential. This is maintained by selective permeability of specific ion channels, both passive and active. The established gradient then creates an electrochemical driving force that, upon activation or opening of channels, allows ions to pass down the electrochemical gradient until either the channels close or the ion reaches its Nernst equilibrium potential (Hille, 2001).

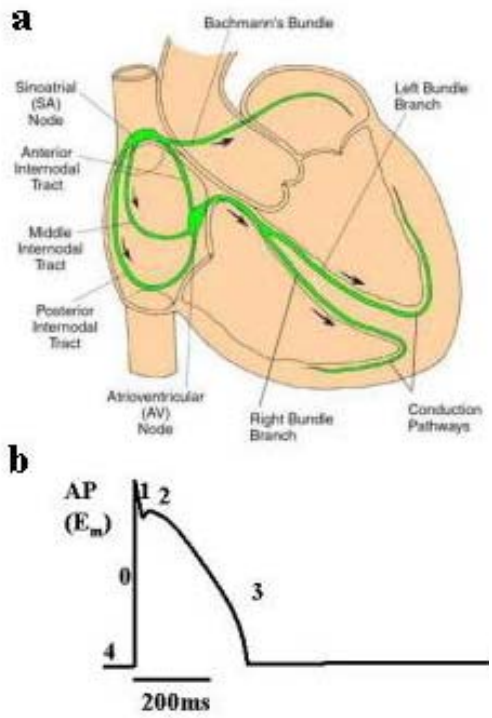


Figure 2. The electrical conduction system of the heart.
A. The electrical impulse within the heart begins at the SA node. It is conducted throughout the atria to the AV node. A slightly delayed impulse is generated at the AV node and propagates through the purkinje fibers then throughout the ventricles as a functional syncytia. (Modified from Wikipedia, September 2006)
B. The five phases of the action potential of a single left ventricular myocyte.

The cardiac AP can be divided into five phases (Figure 2b). In phase four, the resting phase, the membrane potential is about -90mV. This is mainly due to the passive transport of K^+ near its equilibrium potential. Upon stimulation Na^+ channels open, leading to the rapid depolarization of the plasma membrane. In a ventricular myocyte, voltage-gated Na^+ channels are activated to initiate the AP. The rapid depolarization of the sarcolemma activates a select group of K^+ channels leading to an outward I_K at phase 1. At the same time, Na^+ channels enter the inactive state and are therefore unable to open for a given period preventing back propagation of the AP. During this time, LTCCs open leading to influx of Ca^{2+}. The delicate balance between Ca^{2+} influx and K^+ efflux results in the plateau phase (phase 2) of the cardiac AP making it unique in comparison to APs of other excitable cells. Ultimately, Ca^{2+} channels inactivate and the cell repolarizes completely (phase 3).

Ca^{2+} homeostasis

Intracellular Ca^{2+} can regulate multiple diverse physiological processes simultaneously, requiring the precise regulation of local and global Ca^{2+} concentrations. This is achieved by grading Ca^{2+} influx through the plasma membrane, modulating Ca^{2+} release and reuptake into the SR, controlling Ca^{2+} efflux through Ca^{2+} pumps and exchangers and Ca^{2+} buffering by a multitude of Ca^{2+} binding proteins (Bers, 2002b; Hille, 2001). Well established functions of Ca^{2+} in cardiac myocytes include enzyme activation (Maier and Bers, 2002),

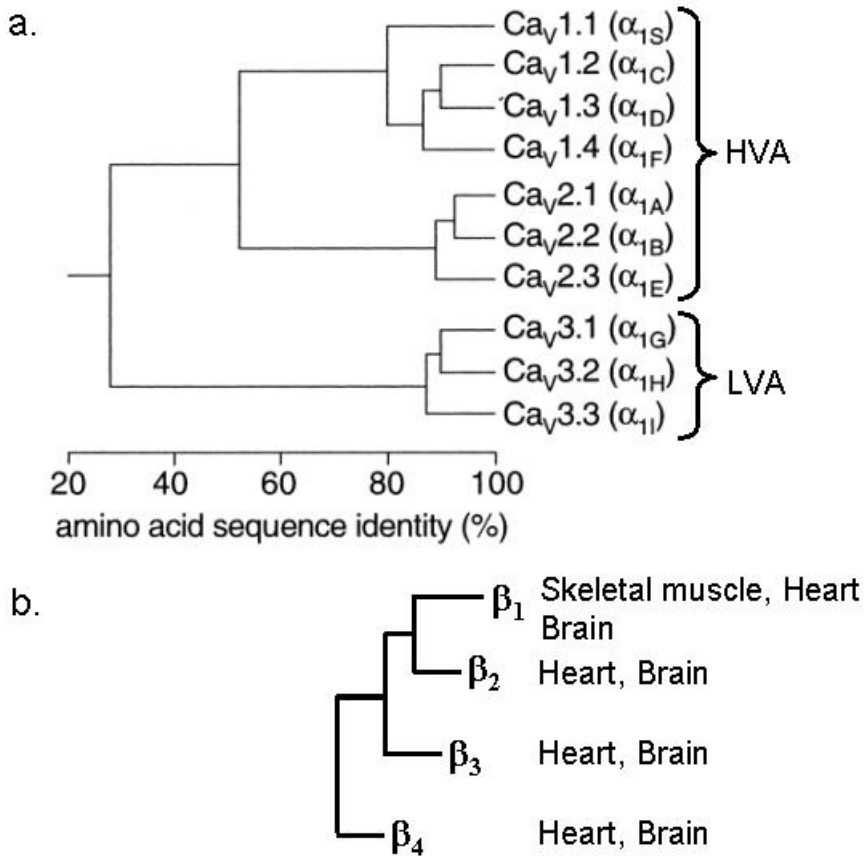


Figure 3. Voltage-gated Ca^{2+} channel dendogram.
A. A dendogram representing the conservation of amino acid sequence among the 10 known pore forming subunits of the VGCC complex. They are divided into three classes based on homology. The Ca_V 1 and 2 groups are high-voltage activated (HVA) and all are thought to directly associate with β subunits while the Ca_V3 group is composed of the low-voltage activated (LVA) class that do not associate with β subunits.
B. Four genes encoding VGCC β subunits and their relative tissue distribution are represented in this dendogram. Any of the four isoforms can associate with the high-voltage activated class of α_1 subunits and differentially modulate the biophysical properties of the channel.

Regulation of gene transcription (Muth et al., 2001) and activation of signaling molecules such as calmodulin (CaM- a small Ca^{2+} binding protein) (Pitt et al., 2001; Zuhlke et al., 1999; Zuhlke et al., 2000). CaMKII is a key CaM-dependent regulator of cardiac Ca^{2+} cycling (Maier and Bers, 2002; Wu et al., 2001a). Alterations in Ca^{2+} transients in cardiac myocytes lead to disease states including arrhythmias and apoptosis (Anderson, 2002; Anderson, 2004; Yang, 2006; Zhu et al., 2003).

Voltage-Gated Calcium Channels (VGCC)

Voltage-gated Ca^{2+} channels are multimeric protein complexes that consist of a large pore-forming α_1 subunit and are usually associated with accessory proteins such as β, $\alpha_2\delta$, and γ depending on the α_1 subunit and cell type (Catterall, 2000). The VGCC act as gatekeepers for Ca^{2+} entry into many cell types including all excitable cells, endocrine cells and other specialized cell types. Regulation of VGCC occurs at many levels and varies depending on the subunit composition. The α_1 subunit forms a pore that opens upon depolarization of the plasma membrane allowing Ca^{2+} to selectively flow down it's electrochemical gradient into the cell.

Ten genes have been identified that encode VGCCs α_1 subunits (Catterall, 2000). They are divided into three groups based on homology and within the groups by their pharmacological properties (Figure 3). The high-voltage activated Ca^{2+} channels include the Ca_V1 (L-type Ca^{2+} channels) and Ca_V2 (N, P/Q and R-type Ca^{2+} channels) groups that all form multimeric protein

complexes. Ca_V3 (T-type Ca^{2+} channels) are low-voltage activated and vary in that they do not typically associate with a β subunit. The α_1 subunit contains the necessary components for activation, inactivation, ion selectivity and drug interactions. The α_1 subunit is a 170-240 kDa protein that contains 24 transmembrane spanning α helices arranged in four homologous repeats (motifs I-IV). The loops connecting the motifs and the C- and N-termini are cytoplasmic, and are known to be important docking and regulatory sites within the α_1 subunit (Anderson, 2001).

$a_2\delta$ subunit

LTCC auxiliary subunits bind to α_1 and regulate expression and functional properties. The $\alpha_2\delta$ subunit is the product of a single gene. The effects on channel kinetics are minor. There are four known genes encoding the $\alpha_2\delta$ subunit ($\alpha_2\delta_{1\text{-}4}$). $\alpha_2\delta$ is almost completely extracellular and may modulate gating effects of β. There is little known about the *in vivo* function of the $\alpha_2\delta$ subunit compared to the α_1 and β subunits (Brickley et al., 1995). Some work using heterologous cell systems suggests that the $\alpha_2\delta$ subunit enhances surface expression of the Ca^{2+} channel complex and enhancing drug binding. The protein product is cleaved into two separate proteins. The α_2 portion is an extracellular protein that is glycosylated. It interacts directly with the extracellular portion of the α_1 subunit. The δ subunit is small containing one transmembrane domain. The α_2 and δ subunits bind to one another via disulfide bonds.

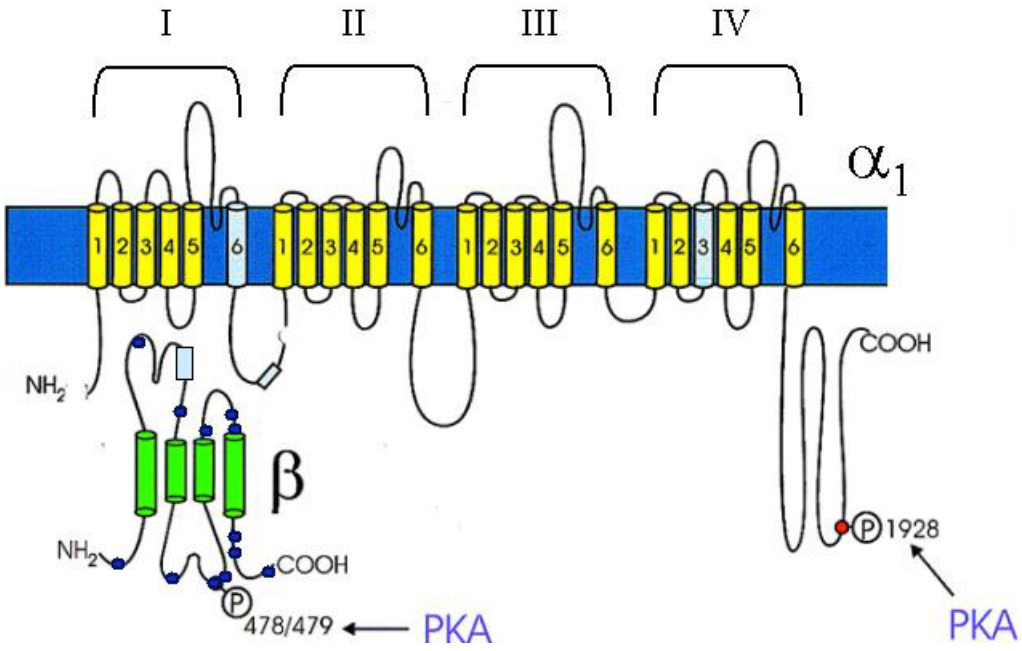


Figure 3. A schematic diagram of the VGCC complex.
The α_1 and β subunits of the VGCC complex are depicted. Also displayed are the PKA phosphorylation sites on α_1 and β. (modified from Catterall 2000)

VGCC regulation

Activation and inactivation of VGCCs are regulated by multiple mechanisms including via accessory proteins such as the β subunit. For LTCC, and P/Q type Ca^{2+} channels it has been shown that Ca^{2+}-dependent feedback via Ca^{2+}/CaM enhances channel inactivation (Lee et al., 2000; Zuhlke et al., 1999; Zuhlke et al., 2000). CaM is a small Ca^{2+} binding protein containing four EF hand motifs each with different affinities for Ca^{2+} that regulates many Ca^{2+} processes. Posttranslational modifications of the proteins within the complex as well as regulation of protein association modify the biophysical properties of the LTCC.

β adrenergic receptor activation has been shown to regulate LTCC by activation of adenylyl cyclase to enhance cAMP concentrations. Increased cAMP leads to the release of A protein kinase (PKA) catalytic subunit and ultimately phosphorylation of the α_{1c} subunit at Ser1928 or, in the absence of the C-terminal portion of the α_{1c} subunit, Ser478/9 of the β_{2a} subunit (Bunemann et al., 1999; Gao et al., 1997a; Gerhardstein et al., 1999). Recent publications suggest additional PKA sites may be the functionally relevant sites (Ganesan et al., 2006). Furthermore, PKA regulation of the cardiac LTCC requires localization via A kinase anchoring protein (AKAP) (Gao et al., 1997b).

Regulation of LTCC by β subunits

There are four genes identified that encode for β, each consists of multiple splice variants and is expressed in a tissue specific manner (Figure 3b) (Perez-Reyes et al., 1992). The β subunit has multiple regulatory roles including

modulating the biophysical properties of the channel and acting as a molecular chaperone that enhances surface expression (Bichet et al., 2000; Birnbaumer et al., 1998; Gao et al., 1997b). β_{2a} causes enhanced I_{Ca} by acting as a chaperone for the α_1 subunits, causes a hyperpolarizing shift in voltage-dependent activation, increases inactivation in whole cell recordings and increases LTCC open probability (P_o) in single channel recordings (Bichet et al., 2000; Hersel et al., 2002). The β isoforms exert a dominant effect when transfected in primary adult cardiac myocytes (Colecraft et al., 2002) and appear to be the "rate limiting" factor for LTCC expression in myocytes (Wei et al., 2000). The regulation of whole cell calcium current (I_{Ca}) is β subunit isoform dependent with $\beta_{2a} \approx \beta_4 > \beta_{1b} > \beta_3$. The most common and well-characterized isoform in cardiac tissue is β_{2a} which is the major focus of these studies.

The β subunits are completely cytosolic. The primary interaction occurs with the α_1 subunit at the I-II linker domain. Recent work disrupting the primary interaction between α and β suggests that secondary interactions are involved in regulating the voltage-dependent properties of the N-type Ca^{2+} channel whereas occupancy is necessary for enhanced channel trafficking by β (Butcher et al., 2006; Leroy et al., 2005). Other sites of interaction include the C-terminus of the CaM binding domains (Zhang et al., 2005a). The α/β complexes of several β subunits bound to α-interacting domain peptides have been crystallized (Chen et al., 2004; Opatowsky et al., 2004; Van Petegem et al., 2004). The crystal structure and sequence homology suggests that β subunits have two major structural domains, a shaker-homology 3 (SH3) domain and a guanylate kinase

like (GK) domain similar to those found in the membrane associated guanylate kinase (MAGUK) family of structural proteins (Takahashi et al., 2004; Takahashi et al., 2005).

Phosphorylation of β_{2a} *in situ* by PKA significantly enhances I_{Ca} in response to β adrenergic receptor stimulation. Key PKA phosphorylation sites on β were identified using a mutagenesis approach (Gerhardstein et al., 1999). Mutation of serines 478 and 479 to alanine rendered I_{Ca} insensitive to PKA, in the presence of truncated α_1 (lacking Ser 1928) (Bunemann et al., 1999). Both CaMKII and PKA cause increases in LTCC openings, suggesting the possibility that they operate through a similar mechanism of β phosphorylation.

CaMKII regulation of cardiac Ca^{2+} homeostasis

CaMKII is a multifunctional kinase that can phosphorylate multiple target proteins sharing consensus motifs containing serine or threonine. CaMKII is able to integrate changes in Ca^{2+} cycling at multiple cell membrane-delimited protein targets. This includes regulation of Ca^{2+} entry into the cell throughLTCC, Ca^{2+} release from the intracellular sarcoplasmic reticulum (SR) stores through ryanodine receptors (RyR) and by SR Ca^{2+} uptake through the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Anderson, 2005). CaMKII is a downstream effector of multiple signaling pathways activated under physiological and pathophysiological conditions (Figure 4). CaMKII expression and activity are up-regulated in structural heart disease (Hoch et al., 1999) (Figure 5). Multiple model systems have been utilized to study the role of

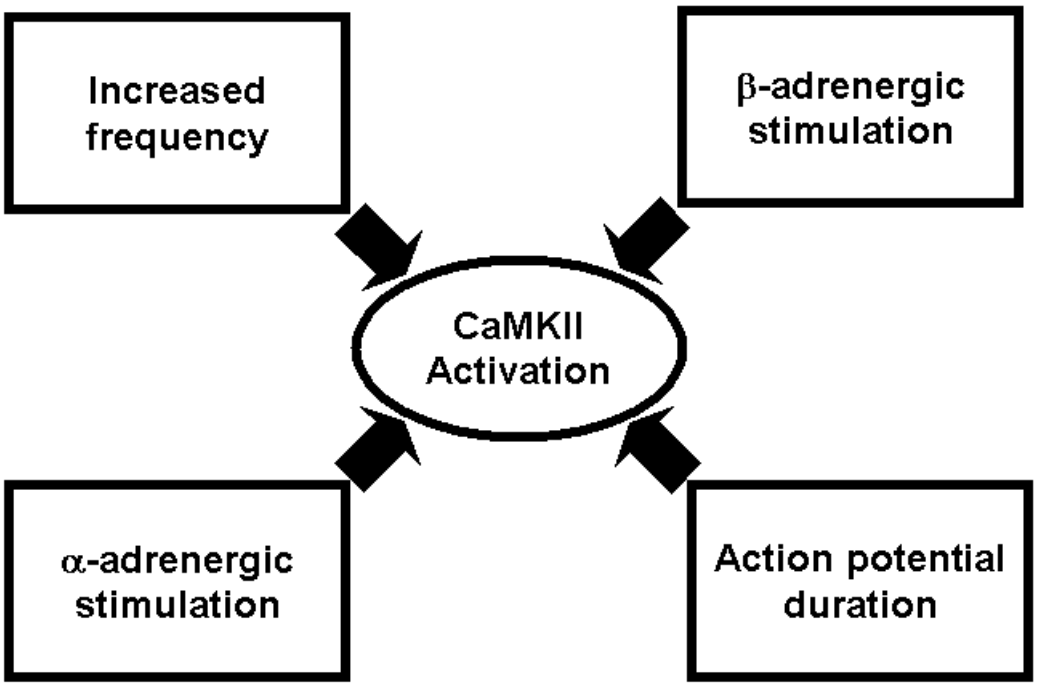


Figure 4. CaMKII activation occurs upon increases in stimulation frequency, α and β adrenergic activation, increased action potential duration and potentially other currently undefined mechanisms.

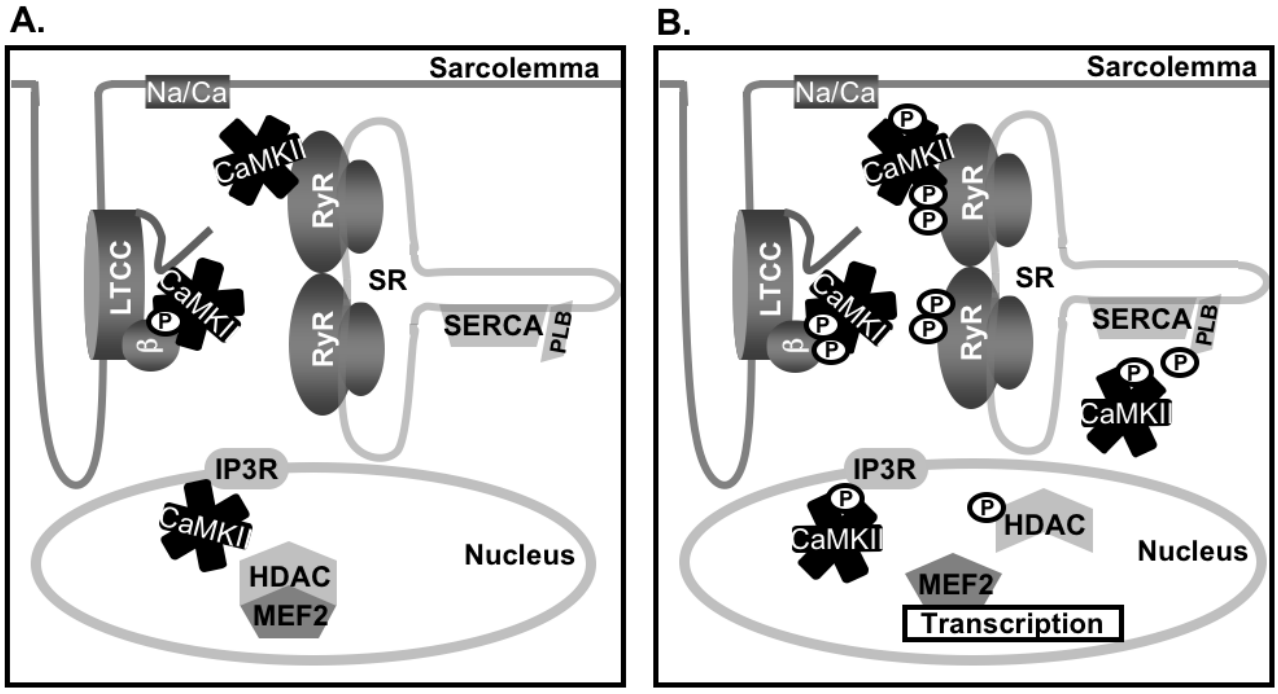


Figure 5. CaMKII binding and phosphorylation targets involved in cardiac Ca^{2+} cycling.
A. Under physiological conditions CaMKII can interact with and regulate multiple partners involved in Ca^{2+} mobilization including the LTCC, RyR and IP_3R. CaMKII can also phosphorylate PLB and potentially SERCA regulating Ca^{2+} reuptake. B. In disease states CaMKII protein expression and activity levels increase with an increase in RyR and PLB phosphorylation and potentially other CaMKII targets.

CaMKII in cardiac disease including transgenic mouse models overexpressing CaMKIIδB (Zhang et al., 2002), CaMKIIδC (Maier et al., 2003; Zhang et al., 2003) and CaMKIV (Passier et al., 2000) as well as transgenic mouse models with universal (Zhang et al., 2005b) and targeted (Ji et al., 2003; Ji et al., 2006) chronic CaMK inhibition. CaMKIV is a monomeric CaM-dependent kinase that is not thought to be expressed in the heart but has been used study the effects of CaMK dependent actions in the heart (Passier et al., 2000). The prominent role of CaMKII in heart disease makes it an attractive candidate for targeted therapy.

CaMKII structure determines function

CaMKII is a dodecameric holoenzyme. Four genes encode CaMKII subunits (α, β, γ and δ), but only γ and δ have been detected in heart (Hagemann et al., 1999; Tobimatsu and Fujisawa, 1989). The catalytic and regulatory domains of all four gene products are about 90% identical. Upon activation the catalytic domain transfers the γ phosphate from ATP to a serine/threonine within the substrate. The regulatory domain binds Ca^{2+}/CaM and contains multiple phosphorylation sites including Thr287. The variable region of CaMKII links the association domain to the catalytic and regulatory domains and is responsible for a majority of the differences between splice variants (Fig.3). The association domain binds other CaMKII association domains (Kolodziej et al., 2000), allowing for assembly of the dodecameric holoenzyme. Variable splicing allows for distinct localization of CaMKII to specific compartments, thus enhancing specificity (Ramirez et al., 1997; Srinivasan et al., 1994). CaMKIIδ was

demonstrated to be the predominant isoform in mammalian heart (Singer et al., 1997). According to studies by Hagemann et.al., δB (also called δ3) predominates in the adult heart while δC (δ2) is abundant in the embryonic heart (Hagemann et al., 1999). The δB isoform is predominantly nuclear due to a nuclear localization sequence in the variable domain, located at the N terminal region of the association domain that directs δB to the nucleus. CaMKIIδC lacks this sequence and so is predominantly resident in cytoplasm. Heteromultimeric complexes form between the different CaMKII isoforms and splice variants. The localization of the holoenzyme complex is determined 'democratically' according to whether the majority of subunits express or lack a nuclear localization sequence (Srinivasan et al., 1994).

In the absence of Ca^{2+}, parts of the regulatory domain bind to the catalytic domain, occluding the binding of nucleotides and protein substrates. This interaction is disrupted by Ca^{2+}/CaM binding to the regulatory domain, presumably due to a change in protein conformation (Rosenberg et al., 2005). Ca^{2+}/CaM activation of CaMKII occurs to different degrees depending upon the frequency, amplitude and duration of Ca^{2+} transients (De Koninck and Schulman, 1998). Thr287 within the regulatory domain of CaMKII is a critical phosphorylation site. Trans-autophosphorylation of Thr287 requires activation of two adjacent subunits (Figure 6). Phosphorylation of CaMKIIα at Thr286 enhances the binding affinity for CaM by 1000 fold from nanomolar to picomolar (Meyer et al., 1992). Phosphorylation at Thr287 also confers 20-80% Ca^{2+}/CaM independent activity, depending on the experimental conditions (Maier and Bers,

2002; Meyer et al., 1992). The ability of CaMKII to sustain its activity through autophosphorylation, even in the absence of elevated Ca^{2+}/CaM, confers remarkable flexibility for extending CaMKII-dependent regulation over time and changes in the frequency of Ca^{2+} transients (Figure 7). Thus, CaMKII is well configured to integrate Ca^{2+} signals to provide feedback regulation of Ca^{2+} and to 'connect' these transients to Ca^{2+}-dependent transcriptional tasks that are important for production and maintenance of the contractile apparatus in cardiac myocytes.

CaMKII localization

In addition to the direct regulation of enzymatic activity by Ca^{2+}/CaM activation and autophosphorylation, CaMKII appears to be dynamically targeted to its substrate in diverse subcellular compartments. Recent studies have demonstrated direct interactions between CaMKII and the RyR (Currie et al., 2004; Hain et al., 1995), the IP_3R (Bare et al., 2005) and α_{1c} subunits of the LTCC complex (Grueter et al., 2006; Hudmon et al., 2005). Indirect evidence also suggests a localized pool of CaMKII exists at the longitudinal SR that regulates Ca^{2+} uptake into the SR (Ji et al., 2003; Ji et al., 2006). Unlike protein kinase A (PKA) which is targeted to specific microdomains by scaffolding proteins known as AKAPs (Gao et al., 1997b), studies involving CaMKII localization suggest CaMKII is targeted by direct interaction with the signaling domains, the predominant cardiac Ca^{2+} channel ($Ca_V1.2$) is highly responsive to

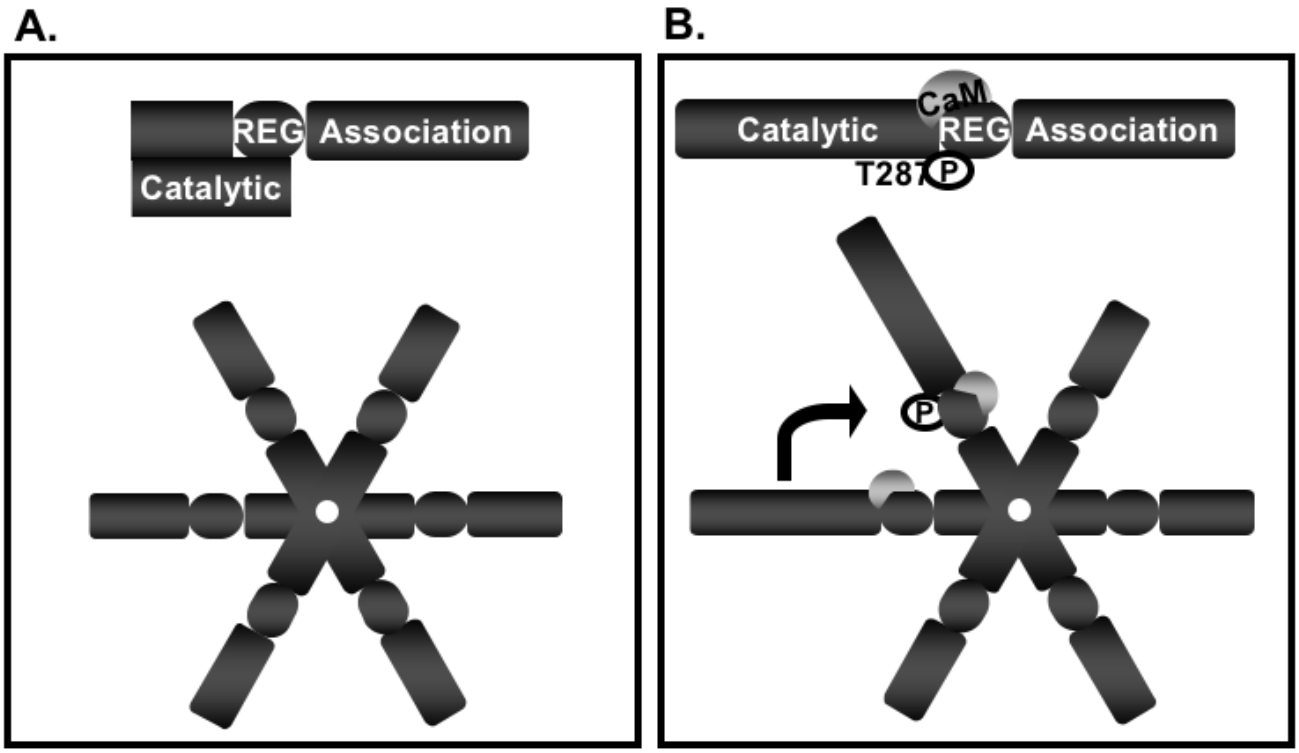


Figure 6. CaMKII activation.
A. Top, a schematic diagram of CaMKII showing the catalytic domain interacting with the regulatory domain in the inactive conformation. CaMKII forms a dodecameric holoenzyme in a stacked hexameric ring conformation (only one hexameric ring is shown) via interactions between the association domains of each monomer as depicted in the bottom panel. B. CaMKII activation via Ca^{2+}/CaM interaction with the regulatory domain, relieving the inhibition. Activation of two adjacent monomers enables trans autophosphorylation at Thr 287. Phosphorylation of Thr287 confers a constitutively active kinase.

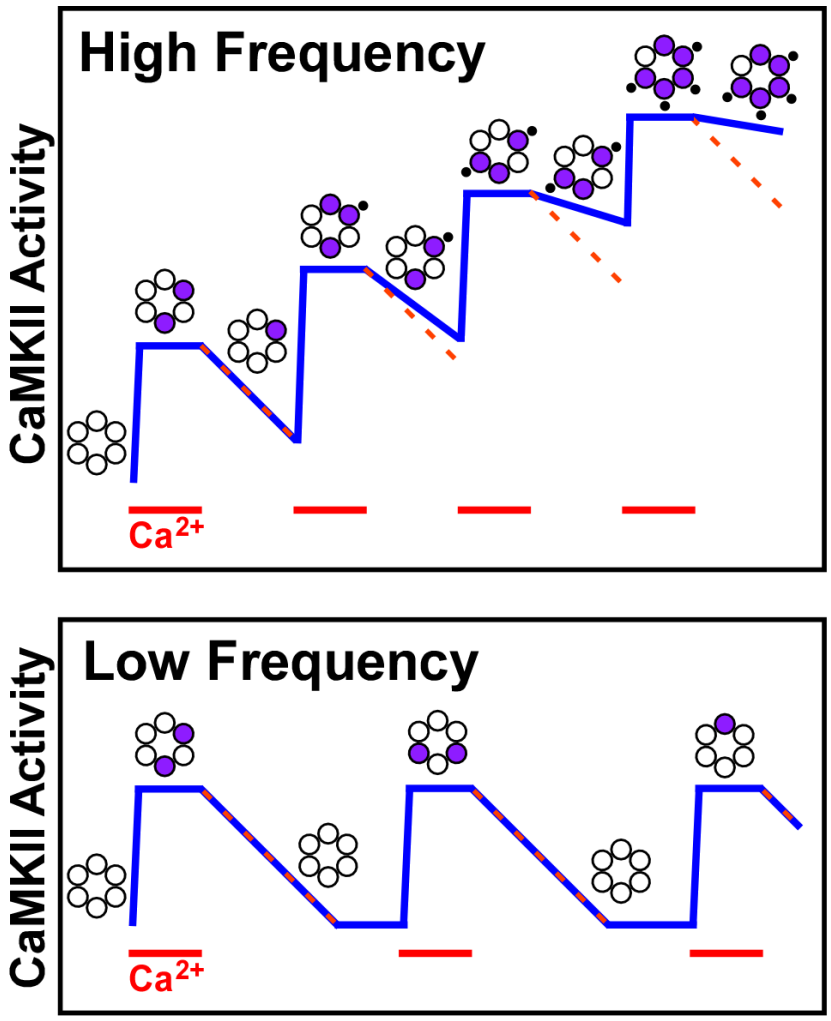


Figure 7. CaMKII activity is dependent on the frequency of Ca^{2+} transients. Inactive CaMKII subunits (open circles) within the holoenzyme (shown as a hexamer for simplicity) become active when bound to CaM (purple) after Ca^{2+} transients (red lines), thus increasing kinase activity (blue line). During high frequency Ca^{2+} transients (above), CaM does not have time to fully dissociate, allowing binding to adjacent subunits and Thr287 autophosphorylation (black dot). This slows the rate of CaM dissociation ("trapping" CaM) and loss of activity (compare to dotted orange line). Low frequency stimulation (below) leaves ample time for CaM dissociation, and thus does not promote Thr287 autophosphorylation or "trapping." (Adapted from (Hudmon and Schulman 2002) and A. J. Robison thesis).

Ca^{2+}_i signals for feedback control. Our laboratory (Dzhura et al., 2000; Dzhura et al., 2003; Wu et al., 2001a; Wu et al., 2001b; Wu et al., 2002; Zhang et al., 2005a) and others (Hudmon et al., 2005; Pitt et al., 2001; Zuhlke et al., 1999) have demonstrated an important role for Ca^{2+}, CaM and CaMKII as feedback mechanisms for LTCC regulation of cardiac contraction and arrhythmias (Dzhura et al., 2000; Kirchhefer et al., 1999; Wu et al., 2001b; Wu et al., 2002). Ca^{2+}-dependent facilitation of I_{Ca} (enhanced peak I_{Ca} and decreased inactivation) was first described in 1982 by Marban and Tsien. CaMKII was first implicated in I_{Ca} facilitation in smooth muscle (McCarron et al., 1992) and later three labs found CaMK inhibitors prevented I_{Ca} facilitation in cardiac myocytes (Anderson et al., 1994; Xiao et al., 1994; Yuan and Bers, 1994). Dzhura et al. demonstrated that phosphorylation by CaMKII drives channels into a high activity gating mode with prolonged openings (Dzhura et al., 2000).

Regulation of voltage-gated LTCC is critical for Ca^{2+} homeostasis and signaling in many normal cells. Defective LTCC regulation contributes to many disease states associated with abnormal levels of Ca^{2+} influx. Several proteins provide for sophisticated regulation of Ca^{2+} influx through the pore-forming α_1 subunit of LTCCs (Arikkath and Campbell, 2003; Bers, 2002a; Bers, 2002b; Dzhura et al., 2000; Pogwizd and Bers, 2002; Walker and De Waard, 1998) including CaMKII. CaMKII preferentially phosphorylates the 55kDa protein vs. the 165kDa protein (the β subunit vs. the α_1 subunit) from skeletal muscle Ca^{2+} channel purifications. PKA, PKC, PKG and casein kinase II all phosphorylate both the α_1 and β subunits but not the $\alpha_2\delta$ or γ subunits (this work was done

before the function of the auxiliary subunits was known) (Jahn et al., 1988). In Chapter III we show that CaMKII regulation of single LTCCs requires the β subunit when transiently expressed in heterologous cells and that mutating β_{2a} Thr498 to alanine ablates CaMKII-dependent regulation of LTCC in heterologous cells and in primary adult cardiac myocytes (Figure 8). Other groups have demonstrated an α_{1c}-dependent mechanism for a form of CaMKII- dependent facilitation in oocytes and voltage-dependent facilitation (Hudmon et al., 2005; Lee et al., 2006). Together these reports suggest multiple modes for CaMKII actions at the LTCC complex.

Ryanodine receptor (RyR)

The second phase of the Ca^{2+} cycle, Ca^{2+} release from internal stores, occurs via Ca^{2+} activation of the RyR. The RyR is a signaling complex with four RyR subunits forming the pore of the channel and a multitude of binding proteins. Included in this constellation of associated proteins are CaM and CaMKII (Currie et al., 2004). Depending on the experimental conditions, CaMKII has been shown to both increase and decrease Ca^{2+} release via RyR (Wehrens et al., 2004; Witcher et al., 1991; Wu et al., 2001a). Endogenous CaMKII is associated with purified RyR, then reconstituted in lipid bilayers, thus decreasing the channel open probability (Hain et al., 1995). Consistent with this effect, the use of CaMK inhibitor peptide AC3I in acutely isolated rabbit cardiac myocytes enhances RyR Ca^{2+} release while blocking CaMK dependent I_{Ca} facilitation, thus enhancing the gain of excitation-contraction coupling. Addition of a constitutively active CaMKII

yielded the opposite result suggesting that CaMK can act as a functional link between LTCC and RyR during excitation-contraction coupling (Wu et al., 2001a). In contrast, acute overexpression of CaMKII in cultured cardiac myocytes by viral transduction enhances the phosphorylation of RyR at both Ser2815 (CaMKII site) and 2809 (also a PKA site) and also increases in Ca^{2+} sparks (Kohlhaas et al., 2006). Chronic overexpression of CaMKIIδC results in altered Ca^{2+} handling with severe cardiac hypertrophy (Maier et al., 2003). Enhanced Ca^{2+} sparks from the RyR and reduced SR content were both observed. This could be a direct result of CaMKII; however, the chronic overexpression of CaMKII also leads to changes in the protein expression level of key Ca^{2+} cycling proteins such as a decrease in SERCA2 and phospholamban (PLB) expression coupled with an increase in the Na/Ca^{2+} exchanger (Maier et al., 2003; Zhang et al., 2003). Thus, it is clear that CaMKII is an important regulator of SR Ca^{2+} release. However, the details of how this process works mechanistically remain to be fully elucidated.

SERCA/PLB

Calcium reuptake into the SR occurs via SERCA on the longitudinal SR. SERCA has been proposed to be directly regulated by CaMKII (Narayanan and Xu, 1997). Other evidence suggests that CaMKII can regulate the frequency dependence of relaxation in the absence of PLB (DeSantiago et al., 2002). However, most studies have focused on SERCA regulation by PLB. SERCA is negatively regulated by PLB (Brittsan and Kranias, 2000). Upon phosphorylation

of PLB by PKA (Bilezikjian et al., 1981) or by CaMKII the inhibition of SERCA is removed allowing for uptake of cytosolic Ca^{2+} back into the SR (Brittsan and Kranias, 2000; Davis et al., 1983; Simmerman et al., 1986). Thr17 (CaMKII site) phosphorylation can occur independently of Ser16 (PKA site) phosphorylation *in vitro* and Thr17 phosphorylation is directly enhanced by increasing stimulation frequency (Hagemann et al., 2000). However, physiological increases in Thr17 phosphorylation probably follow catecholamine-stimulated increases in chronotropy and inotropy (Luo et al., 1998). Thus, Thr17 phosphorylation potentially correlates with an adaptive response to sudden changes in heart rate. Chronic CaMKII inhibition reduces the variability of Ca^{2+} induced Ca^{2+} release in cardiac myocytes by regulation of both SR Ca^{2+} release from stores and Ca^{2+} reuptake by SERCA (Wu et al., 2006b; Wu et al., 2006c). As a result of CaMKII inhibition, PLB Thr17 phosphorylation is significantly reduced (Wu et al., 2006c). Transgenic mice with targeted inhibition of CaMKII at the longitudinal SR show a decrease in PLB Thr17 phosphorylation. These mice exhibit dilated heart failure when stressed by gestation and parturition (Ji et al., 2003), but this result should be interpreted cautiously because the targeting strategy requires over-expression of protein in the SR membrane and no model has yet been developed to control for potential side effects of this strategy. Further studies in these mice also suggest that CaMKII activity at the longitudinal SR contributes to cardiac contractility and Ca^{2+} handling. The rate of contraction and relaxation in whole heart and in isolated myocytes is decreased (Ji et al., 2006).

Inositol 1,4,5-Triphosphate Receptor (IP_3R)

The IP_3R is a Ca^{2+} channel activated by IP_3 and Ca^{2+}. It is localized on intracellular membranes, the nuclear membrane (Wu et al., 2006a) and SR in neonatal cardiac myocytes (Mohler et al., 2004a) and forms a tetrameric complex similar to the RyR. IP_3R2 is the predominant isoform found in the heart (Lipp et al., 2000). The expression of the receptor complex in cardiac myocytes is about 50 fold lower than RyR. The function of the IP_3R in the heart is still being defined. In contrast to RyR, IP_3R are not thought to play a major role in beat-to-beat Ca^{2+} cycling (Go et al., 1995; Marks, 2000). However, studies have suggested a role for IP_3R regulation of transcription in the heart (Go et al., 1995; Marks, 2000). It has been reported that in heart disease IP_3R are up-regulated and may be involved in altered Ca^{2+} homeostasis and cardiac arrhythmias (Mackenzie et al., 2002). IP_3Rs have been shown in one study to localize to the nuclear envelope where they may regulate Ca^{2+} influx into the nucleus to regulate gene transcription by activation of CaMKII. CaMKII can co-precipitate with the IP_3R and the IP_3R are a CaMKII substrate (Bare et al., 2005). IP_3R signaling activates CaMKII by releasing Ca^{2+} in the immediate vicinity of the nucleus to phosphorylate histone deacetylase 5 (HDAC5) thus regulating transcription in a manner independent of the cyclic Ca^{2+} oscillations from beat to beat (Wu et al., 2006a). Together these studies provide evidence that CaMKII localization is necessary for efficient substrate recognition and signal specificity.

CaMKII regulation of myocyte enhancer factor 2 (MEF2) activity

Ca^{2+} signaling requires precise mechanisms to discriminate between changes in local Ca^{2+} concentrations. CaMK has been implicated in activation of a fetal gene program via MEF2 signaling (Blaeser et al., 2000; Passier et al., 2000). MEF2 regulates structural genes and genes involved in growth, stress response and apoptosis. Mice overexpressing CaMKIV have a 100 fold increase in MEF2 activity when interbred with transgenic mice expressing a MEF2 sensor (Passier et al., 2000). The pathway mediating CaMKII-dependent increases in MEF2 transcription activity involves CaMKII interaction with class II histone deacetylases (HDACs). HDAC4 and five are transcriptional repressors that are targeted by multiple serine/threonine kinases including CaMKII acting at two conserved serine residues. Phosphorylation of HDACs at these serines creates a binding site for 14-3-3 protein that occludes a nuclear retention signal to favor movement of HDAC to the cytoplasm. Since HDAC is a MEF2 repressor, prevention of HDAC binding with or without export to the cytoplasm results in enhanced MEF2 activity (McKinsey et al., 2000; Youn et al., 2000). Specifically, CaMKII binding to and signaling via HDAC4 results in hypertrophy (Backs et al., 2006). Thus CaMKII acts as an interpreter and a modulator of local Ca^{2+} concentrations that regulate long term changes in cardiac myocyte gene expression.

Pathways mediating CaMKII activation

During the cardiac Ca^{2+} cycle local intracellular Ca^{2+} concentration ranges from ~0.1μM at diastole to over 100μM in the microdomain of the LTCC and RyR during systole (Bers and Guo, 2005). It was demonstrated that CaMKII has a tonic level of activity under basal conditions and that activated (Thr287 autophosphorylated) CaMKII localizes to the sarcolemma with a T-tubule staining pattern (Xiao et al., 1994). The major questions remaining include: under what physiological or pathological conditions is CaMKII activated and what is its function within specific pathways (Figure 4)? Many studies have begun to address these questions. These include experiments showing that increased frequency and action potential duration both enhance CaMKII activity (Anderson et al., 1998; De Koninck and Schulman, 1998; Wu et al., 1999b).

The β-adrenergic system is the most prominent regulator of cardiac function and 'β blockers' have been the major therapy for treating patients with structural heart disease and myocardial dysfunction (Gottlieb, 1998; Tendera and Ochala, 2001). The traditional pathway mediating β-adrenergic signaling involves activation of G_s-protein coupled receptors. Activation of G_s leads to activation of adenylyl cyclase to enhance cAMP production and ultimately activate PKA. PKA targets many of the same proteins in the Ca^{2+} cycle as CaMKII, leading to enhanced Ca^{2+} mobilization. Recent studies have revealed other means of β-adrenergic signaling via CaMKII (Wang et al., 2004; Zhang et al., 2005b; Zhu et al., 2003). Our recent findings support the hypothesis that CaMKII is a key downstream effector of the β-adrenergic receptor signaling

cascade. It is intriguing that CaMKII inhibition does not appear to affect the "fight or flight" responses to βAR activation. However, chronic inhibition of CaMKII in AC3I mice and acute inhibition using KN93 also protects the mice from cardiomyopathic responses to chronic βAR stimulation. Transgenic over-expression of AC3I, a CaMKII inhibitory peptide, mitigates the deleterious impact of myocardial infarction on left ventricular function (Zhang et al., 2005b). These studies show that CaMKII activity directly contributes to loss of Ca^{2+} homeostasis in two cardiac disease models associated with βAR activation. Zhu et al studied the effects of β_1 adrenergic activation on apoptosis in the presence of several PKA inhibitors and found that activation of CaMKII not PKA was responsible for increased programmed cell death during excessive isoproterenol *in vitro* (Zhu et al., 2003). They found that CaMKII activation was required for this response. Subsequent findings support CaMKII activation upon β_1AR stimulation as an integral part of the enhanced cardiac contractility (Wang et al., 2004). Our group found that CaMKII inhibition protected against apoptosis *in vivo* during myocardial infarction or excessive β_1AR stimulation with isoproterenol (Yang, 2006). These studies suggested that the proapoptotic actions of CaMKII were related to its regulation of SR Ca^{2+} content because the benefits of CaMKII inhibition for reducing apoptosis were lost when AC3I mice were bred into a PLB null background with SR Ca^{2+} overload.

The α_1-adrenergic signaling cascade activates CaMKII by a mechanism requiring PKC activation. The functional outcome of α-adrenergic stimulation of CaMKII is enhanced I_{Ca} from LTCC and an increase in activated CaMKII

localization at the T-tubules (O-Uchi et al., 2005). Activation of the α-adrenergic system also turns on genes involved in cardiac hypertrophy and the response is prevented by CaMKII inhibition (Ramirez et al., 1997). This suggests that CaMKII activation by the α-adrenergic system could potentially regulate local Ca^{2+} signaling as well as global gene expression.

CaMKII as a signal in structural heart disease: fulfilling Koch's postulates

Robert Koch established a systematic guideline in the 1890's to identify a microorganism as the source of a disease (Koch, 1893). Simply stated, the microorganism must be found in all cases of the disease. It must be isolated from the host and grown in pure culture. It must reproduce the original disease when introduced into a susceptible host and it must be found in the experimental host so infected (Chien, 2000). Koch's postulates can be adapted and partially applied to structural heart disease (Chien, 2000). Molecules fulfilling Koch's postulates would first need to have altered expression or be misregulated in disease. In the case of CaMKII, knocking out the protein or inhibiting its activity would be protective against the disease. Reintroducing an excess or constitutively active mutant would lead to the disease in a previously normal tissue. Finally, over-activity of CaMKII would then be verified in the host tissue. Much work has been published implicating CaMKII as an integral part of many forms of cardiomyopathy in human and animal models.

CaMKII expression and activity are increased in patients with end stage heart failure (Hoch et al., 1999; Kirchhefer et al., 1999) and in animal models (Colomer

and Means, 2000; Wu et al., 2002) of heart disease. Transgenic mice over-expressing CaMKIIδc develop dilated cardiomyopathy and sudden death (Zhang et al., 2003). According to Koch's postulates, the cause of the disease must be isolated from the experimental host. In order to isolate CaMK as a significant focal point in cardiomyopathy, a transgenic mouse was engineered with a CaMK inhibitory peptide (AC3I). These mice exhibit a significant cardioprotective effect following myocardial infarction and chronic β-AR stimulation (Zhang et al., 2005b). Transgenic mice over-expressing CaMKIV in heart have severe cardiomyopathy, and addition of constitutively active CaMKII "restores" I_{Ca} facilitation (Wu et al., 1999a). While Koch's postulates for infectious disease do not provide a completely parallel analysis for the study of structural heart disease, it is becoming increasingly clear that CaMKII is a critical cardiomyopathic signal in structural heart disease in patients and in numerous animal models of cardiomyopathy (Zhang and Brown, 2004).

CaMKII in structural heart disease

Structural heart disease is characterized by 1) electrical instability and arrhythmias, 2) myocardial dysfunction, and 3) myocardial hypertrophy and chamber dilation (Jessup and Brozena, 2003). One possibility is that targeted inhibition of CaMKII could improve the fundamental changes in structural heart disease (Anderson, 2005).

Electrical changes are seen in heart disease due to remodeling of Ca signaling proteins and other ion transport proteins. In heart disease CaMKII

protein and activity levels are increased along with changes in ion channel expression. The changes in proteins involved in electrical propagation and Ca^{2+} cycling may result in an increased probability of developing arrhythmias (Anderson, 2004; Shah et al., 2005). In addition, structural heart disease creates a substrate that favors arrhythmias. The formation of a physical barrier for electrical conduction along with remodeling of key Ca^{2+} regulatory proteins and increased neurohormonal stimulation combine to provide favorable circumstances for arrhythmias to occur. These include AP prolongation (electrical remodeling) and prolongation of the Ca^{2+} transient ('Ca^{2+}' remodeling).

Due to the high electrical resistance of the cell membrane at the plateau phase of the cardiac AP, slight changes in I_{Ca} or I_k lead to dramatic changes in the AP, including early-after depolarization's (Keating and Sanguinetti, 2001). Chronic CaMKII over-expression leads to electrical remodeling and increased susceptibility to sudden death (Maier et al., 2003) and CaMKII is proarrhythmic in various models of AP prolongation in structurally normal hearts and isolated cardiac myoctyes (Anderson et al., 1998; Gbadebo et al., 2002; Kirchhof et al., 2004; Pak et al., 1997; Wu et al., 1999b). CaMKII actions at LTTCs are particularly important under voltage and SR Ca^{2+} release conditions present at the action potential plateau (Wu et al., 2004). Enhanced RyR Ca^{2+} leak or general increases in intracellular Ca^{2+} concentration due to altered CaMKII activity can lead to increased Na^{+}/Ca^{2+} exchanger activity, thus enhancing the probability of producing delayed after-depolarizations. CaMKII inhibition reduces the Na/Ca^{2+} exchanger current (also called the transient inward current) by

reducing SR Ca^{2+} release under conditions of cellular Ca^{2+} overload (Wu et al., 1999b). Because CaMKII can enhance I_{Ca} it was thought to potentially play a role in mediating early-afterdepolarizations. Indeed, in studies where early-afterdepolarizations were induced, CaMKII inhibition significantly decreased the occurrence of early-afterdepolarizations (Wu et al., 1999a; Wu et al., 1999b). Taken together, these diverse mechanisms for CaMKII signaling in heart disease conspire to build proarrhythmic inward currents under conditions of adverse electrical and Ca^{2+} remodeling that are a fundamental characteristic of structural heart disease.

Other triggers for arrhythmias are neurohormonal activation of signaling pathways such as the β-adrenergic pathway that leads to increased heart rate and frequency. Activation of this pathway also recruits CaMKII and enhances Ca^{2+} cycling (Zhang et al., 2005b; Zhu et al., 2003). These findings suggest to us that the efficacy of βAR antagonist drugs ('β blockers') in preventing sudden cardiac death may be in part related to their inhibition of CaMKII activity.

Strategies for targeting CaMKII

Protein kinases are second only to G-protein coupled receptors as therapeutic drug targets. They have been highly researched in the cancer field and now comprise as much as 20-30% of the research at many pharmaceutical companies (Cohen, 2002). Most kinase inhibitors, however, target the ATP binding domain, thus limiting the identification of selective inhibitors for specific kinases (Cohen, 2002). Importantly, the structural information that is steadily

becoming available for many kinases provides useful clues for development of more specific inhibitors. Among the recently crystallized structures is a structure of the regulatory and catalytic domains of CaMKII (Rosenberg et al., 2005). This new information suggests that specific inhibitory agents could be developed by allosterically altering the ATP binding pocket. As pointed out by Cohen, critical studies that remain are a detailed analysis of inhibitors and their effects on catalytic and regulatory properties (Cohen, 2002).

CaMKII is emerging as an attractive candidate for drug therapy in structural heart disease and arrhythmias. However, there are many caveats to targeting this multifunctional kinase. Among the many known roles for CaMKII is gene regulation and molecular memory (Colbran and Brown, 2004; Lisman et al., 2002). New studies of CaMKII target proteins are providing evidence for the mechanism of CaMKII action. An attractive model may be to target CaMKII substrates that are critical components of Ca^{2+} cycling, thus taking a more specific approach to regulating CaMKII in Ca^{2+} signaling.

CaMKII inhibitors such as KN62 and KN93, whose mode of action is to bind to the Ca^{2+}/CaM domain and inhibit CaMKII activation have provided useful information on the function of CaMKII in cardiac myocytes. However, these data should be interpreted with care because of the effects of these drugs on multiple ion channels (Anderson et al., 1998). More selective CaMKII inhibitors are needed.

Endogenous inhibitors of CaMKII have been identified: the kinase itself contains an autoregulatory domain that reversibly binds to the catalytic domain

and inhibits the kinase (as discussed previously). A brain-specific protein CaMKIINβ was identified by yeast-2-hybrid and found to selectively inhibit CaMKII (Chang et al., 1998). Another endogenous inhibitor of CaMKII is the NR2B subunit of the NMDA receptor (a glutamatergic ligand-gated ion channel). It contains a motif mimicking the autoregulatory domain of CaMKII that directly interacts with the kinase (Bayer et al., 2001; Robison et al., 2005; Strack et al., 1997; Strack and Colbran, 1998; Strack et al., 2000a) and this interaction inhibits CaMKII activity *in vitro.* Zhang et. al. showed marked protection of cardiac function in a structural and neurohumoral model by chronic inhibition of CaMK throughout the cell (Zhang et al., 2005b). Some studies have been done looking at the effect of inhibition of localized pools of CaMKII; however, more work is needed (Ji et al., 2003; Ji et al., 2006).

The use of viral mediated transduction of specific gene products or mutants has become a valuable tool for identifying the role of CaMKII target proteins *in vivo* (Colecraft et al., 2002; Kohlhaas et al., 2006; Mohler et al., 2003; Wu et al., 2006c). Work has been done demonstrating the possibility of injecting virus directly into live animals and studying the effect on whole heart physiology (Gregorevic et al., 2004). Viral approaches provide an alternative approach to production of transgenic mice and are more easily used to study effects in other animal models such as rabbits whose cardiac physiology is more closely related to humans.

Summary

In cardiac disease, it has been well documented that there is an underlying mishandling of Ca^{2+}. Whether an alteration in Ca^{2+} cycling is a cause or effect of the disease is still under investigation. Importantly, key Ca^{2+} signaling molecules such as the multifunctional CaMKII are emerging as focal points for studying cardiac disease. CaMKII has been identified as a determinant of the severity of the outcome of a structural and neurohormonal model of cardiomyopathy with inhibition of CaMKII having a significant functional benefit. Further studies are needed to identify the mechanisms for CaMKII regulation of the proteins regulating Ca^{2+} cycling and the contribution of these points to cardiac disease. Increased effort to develop novel clinically-relevant strategies for CaMKII inhibition is clearly warranted.

CaMKII facilitates cardiac I_{Ca} in response to multiple forms of activation including repeated stimulation (Figure 4). The proposed physiological role of increased I_{Ca} is to increase Ca^{2+} influx, therefore enhancing the force of contraction upon increased heart rate. The focus of my research has been to elucidate the mechanism for CaMKII dependent regulation of cardiac LTCCs, focusing on the involvement of the β subunit. The following chapters discuss the important findings of my research. Chapter III focuses on the identification and characterization of CaMKII regulation of cardiac LTCCs mediated by phosphorylation of β_{2a} Thr498 in heterologous cells and adult cardiac myocytes. Also described in this chapter is the identification of the β_{2a} subunit as a CaMKII

associated protein (CaMKAP). Chapter IV continues to delineate the mechanism for CaMKII interaction with β_{2a} as well as other β isoforms. The final chapter summarizes the work completed as well as provide a roadmap for the many new exciting avenues of research that have resulted from the findings within this thesis.

Hypothesis

We hypothesized that CaMKII regulates cardiac LTCC function via the β subunit. Four aims were designed to address this hypothesis.

Specific Aims

Aim 1 determined the CaMKII phosphorylation sites on the β subunit using biochemical and proteomic approaches.

Aim 2 tested the functional consequence of β subunit phosphorylation by CaMKII in a model heterologous cell system.

Aim 3 tested the regulation of LTCC by CaMKII phosphorylation of the β subunit in adult cardiac myocytes.

Aim 4 tested the interaction between CaMKII and β subunits.

CHAPTER II

MATERIALS AND METHODS

Generation of plasmid constructs

The open reading frame of rat β_{2a} (Accession Number M80545) was amplified by PCR and ligated into pGEX-4T1 (Amersham Pharmacia Biotech), pFLAG-CMV-2 (Sigma-Aldrich) pIRES (Clontech), and pLenti (Invitrogen) vectors. Other vectors used include pcDNA3 (Invitrogen) containing murine CaMKIIα a generous gift from Y. Nikandrova, Vanderbilt University) and pGW1H (British Biolabs) containing α_{1c} (Accession Number X15539, a generous gift from Dr. T. Kamp, University of Wisconsin). The cDNAs encoding β_{2a} mutants were made using the QuikChange Mutagenesis kit, essentially as described by the manufacturer (Stratagene). DNA sequences of all mutated and wild type sequences were confirmed.

GST fusion protein expression and purification

BL21DE3 pLysS E.coli were transformed with pGEX-4T1 constructs, grown at 37°C to a density of >0.75 and GST fusion protein expression was induced with IPTG (1 mM final). Cells were harvested 3 hours later and proteins were purified using glutathione-agarose, dialyzed into storage buffer (50 mM

Tris-HCl pH 7.5, 200 mM NaCl, 0.05% Triton X-100, 0.05 mM benzamidine, 0.125 mM PMSF, 0.125 mM EDTA) and stored at -80°C. Protein concentrations were determined by Bradford assay (BioRad), using bovine serum albumin as standard.

CaMKII purification and autophosphorylation

Recombinant rat CaMKIIδ_2 or mouse CaMKIIα purified from baculovirus-infected Sf9 insect cells was autophosphorylated at Thr287 ([P-T287]) or Thr286, respectively, using ATP or [γ-^{32}P]ATP, essentially as described previously (Strack et al., 2000b).

Glutathione-agarose co-sedimentation assay

GST fusion proteins (300 pmol) were incubated at 4°C for 1 hour with CaMKIIδ_2 or [P-T287]CaMKIIδ_2 (100 pmol subunit) in 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 1 hour. Resin was collected in a microcentrifuge (1 min, 4,500 *g*) and washed 4 times in Binding Buffer. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and then immunoblotted for CaMKII.

CaMKII plate binding assays

GST fusion proteins in plate-binding buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 100 mM EDTA, 5 mM 2-mercaptoethanol, 0.1 % Tween-20, 5 mg/ml bovine serum albumin) were incubated at 4°C in glutathione-coated wells for 18-24 hours (5 pmol in 0.2 ml). After 3 washes with buffer, wells were incubated at 4°C with [^{32}P-T287]CaMKIIδ_2 (0.2 ml, indicated subunit concentration) for 2 hours then washed (8 times, 0.2 ml ice-cold buffer). Bound kinase was quantified in a scintillation counter.

CaMKII gel overlays

GST fusion proteins (50 pmoles) 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 in 50 mM Tris-HCl, pH 7.5 containing 200 mM NaCl, 3% (v/v) Tween-20, 5% (w/v) milk, and then incubated at 4°C for 2 hours with [^{32}P-T287]CaMKIIδ_2 (100 nM). After washing, membranes were exposed using a phosphoimager to quantify the bound CaMKII.

Kinase assays

GST-β_{2a} (wild-type or mutated) was incubated at 30 °C in 50 mM HEPES, pH 7.5, 10 mM magnesium acetate, 1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.4 mM [γ-^{32}]ATP (≈ 500 cpm/pmol) or 0.4 mM ATP containing either CaMKII (at the indicated subunit concentration), 0.5 mM calcium chloride,

1 μM calmodulin, or PKA catalytic subunit (generous gift from Dr. J.D. Corbin, Vanderbilt University). PKA and CaMKII displayed similar specific activities (2-3 μmol/min/mg) toward model peptide substrates (100 μM Kemptide and 20 μM syntide-2, respectively). Phosphorylation stoichiometries were determined by spotting aliquots on P81 phosphocellulose paper and washing prior to quantifying ^{32}P incorporation using a scintillation counter. Counts detected in control reactions containing GST alone rather that GST-β_{2a} was subtracted to correct for kinase autophosphorylation. Alternatively, reactions were quenched by adding 4x SDS sample buffer: gel samples were resolved by SDS-polyacrylamide gel electrophoresis. Coomassie Blue-stained gels were dried and exposed to film or a phosphoimager followed by densitometry using BioRad imaging software.

CaMKII inhibition by β_{2a}

CaMKII phosphorylation of a synthetic peptide, syntide 2, was assayed in the presence of various concentrations of GST-β_{2a} wild-type and mutants or GST-NR2B wild type (1180-C-terminus) and Ser1303Ala (1190-1339) . Assays were performed using preautophosphorylated kinase in the presence of Ca^{2+}/CaM. The assay contained 0.4mM [γ-^{32}P]ATP and 0.2 mM syntide-2 .

Phosphoamino acid analysis

Radiolabeled GST-β_{2a} (WT or 410-604) was excised from dried Coomassie Blue-stained SDS-polyacrylamide gels. Protein was extracted in 3.2 M ammonium bicarbonate, 2-mercaptoethanol (5%, v/v) and 0.1% SDS,

precipitated using ice-cold trichloroacetic acid, and then partially hydrolyzed with 5.7 M HCl at 110°C for 60min. Samples were mixed with non-radioactive phospho-serine and phospho-threonine in a pH 1.9 buffer (2.2% (v/v) formic acid, 7.8% (v/v) glacial acetic acid), spotted on thin layer cellulose plates and then separated in one dimension by thin-layer electrophoresis using a Hunter Thin-Layer Peptide Mapping system. Cellulose plates were stained with ninhydrin to detect nonradioactive standards and then exposed to X-ray film.

Mass spectrometry

Phosphorylated GST-β_{2a} was re-purified from the phosphorylation mixture using glutathione-agarose. Excess glutathione was removed using a 30kDa Ultrafree MC regenerated cellulose filter (Millipore) and protein was digested (Manza et al., 2005) using either trypsin (18 hr, 37°C) or chymotrypsin (18 hr, room temperature). Peptides were separated for LC-MS/MS analysis by HPLC using a capillary column (Monitor C18, 100 μm x 11 cm, 5 micron, 100 Å, Column Engineering). The flow rate was 0.7 μl min^{-1} with a gradient from Solvent A (0.1% formic acid in H_2O) to solvent B (0.1% formic acid in acetonitrile) as follows: 0-3 min, linear gradient from 0-5% B; 3-5 min, 5% B; 5-50 min, linear gradient to 50% B; 50-52 min, linear gradient to 80% B; 52-55 min, linear gradient to 90% B; 55-56 min, 90% B. MS/MS scans of peptides were acquired using a ThermoFinnigan LTQ linear ion trap mass spectrometer equipped with a ThermoFinnigan Surveyor LC pump, NanoSpray source (Thermo Electron), and Xcalibur 1.4 instrument control and data analysis software, with an isolation width

of 3 m/z, an activation time of 30 ms, and activation Q of 0.250 and 30% normalized collision energy using 1 microscan and ion time of 100 for each MS/MS scan. The mass-spectrometer was tuned prior to analysis using the synthetic peptide TpepK (AVAGKAGAR), typical tune parameters were as follows: spray voltage of between 1.8 KV, a capillary temperature of 150ºC, a capillary voltage of 50V and tube lens 100V. Tandem MS analysis was performed using data-dependent scanning in which one full MS spectra (mass range of 400-2000 amu) was followed by 3 MS/MS spectra. Peptides and modifications were identified using both the SEQUEST algorithm with SEQUEST Browser software (Thermo Electron, San Jose, CA) and P-Mod software (Hansen et al., 2005). Candidate modifications found by software were verified by visual inspection of corresponding spectra.

Co-immunoprecipitations from HEK293 cells

HEK293 cells (10cm dish) transfected using Fugene6 with vectors (4 μg each) containing FLAG-β_{2a} (WT or T498A), CaMKIIα, and/or empty vector were lysed in 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl, 1 mM EDTA, 10 mM NaF, 1 μM microcystin-LR, 10 mM PMSF, 1 mM benzamidine, 1X general protease inhibitor cocktail plus 1 μg/ml pepstatin (Sigma). Aliquots of the cell lysates, FLAG immunoprecipitates (40 μl: Sigma) and immune supernatants were immunoblotted for FLAG proteins and CaMKII using enhanced chemiluminescence development.

Western blot

Western blots were done using standard procedures. Briefly, membranes were blocked in 5% milk in TTBS, then incubated with primary antibodies overnight at 4ºC. The membranes were washed six times for at least 5 minutes each followed by incubation of the appropriate HRP conjugated secondary antibody at room temperature for one hour followed by washing. The membranes were developed using enhanced chemiluminescence.

tsA201 cell culture and transfection

The tsA201 cells (modified HEK293 cells stably expressing small T antigen) were maintained at 37°C in 5% CO_2 in DMEM media supplemented with 10% fetal bovine serum and 1% penicillin (100 U/ml)/streptomycin (100 μg/ml). Cells were transfected with pGW1H-α_{1c} (1 μg) with or without pIRES-β_{2a} (2 μg) using Lipofectamine (Invitrogen) according to the manufacturer's protocol. After 24 hours, cells were placed in a 28°C incubator.

Single channel recordings

Currents were recorded from excised patches of tsA201 cells (48-96 hours post-transfection) in the inside-out configuration at depolarizing steps from -70mV to 0mV for 200 ms. The bath solution contained 150mM KCl, 10 mM EGTA, 10 mM HEPES, 7.5 mM or 11 mM $CaCl_2$, 5.5 mM glucose, 1mM EDTA, 0.01 mM ATP; the pH was adjusted to 7.4 with 10 N KOH. The pipette solution contained 110 mM $BaCl_2$, 5 mM HEPES, 0.03 mM TTX; the pH was adjusted to

7.4 with Trizma base. Samples were taken at 20 kHz and low-pass filtered at 2 kHz using 4 pole Bessel. Only patches containing a single Ca^{2+} channel were analyzed.

Lenti virus production

DNA inserts containing eGFP-IRE-FLAGβ_{2a} (WT and T498A) and vGFP were inserted into the pLenti6 plasmid and then co-transfected into 293FT cells with three viral packaging plasmids (pLP1, pLP2, and pLP/VSVG) using Lipofectamine 2000 (Invitrogen) (Mohler et al., 2004b). Culture medium was replaced after 24 hours. After 48 hours the virus containing media was removed and centrifuged to remove cellular debris.

Myocyte isolation, culture and infection

Adult Sprague-Dawley rats were anesthetized using Avertin (0.2 ml/10 g,IP) with heparin. Hearts were excised and ventricular myocytes isolated by enzymatic digest with Collagenase type II and Proteinase using a Langendorrf perfusion apparatus. Isolated cells were filtered and washed 3 times in MEM 1081 culture medium (Sigma) containing ITS liquid media supplement (Sigma), penicillin (100 U/ml)/streptamycin (100 μg/ml), 4 mM $NaHCO_3$, 2.5% fetal bovine serum, and adjusted to pH 7.4 with NaOH. Myocytes were plated at low density on laminin coated plates for 1 hour at 37°C at 95% O_2 and 5% CO_2. Cells were washed briefly in phosphate-buffered saline then culture medium was added.

Virus (20 μl) with a similar multiplicity or infection was added and cultures were maintained for 15-24 hours before analysis.

Immunofluorescence

Briefly, cultured adult cardiac myocytes were washed with warm phosphate-buffered saline (PBS, pH 7.4) and fixed in 2% paraformaldehyde for 20 minutes (37°C). Cells were blocked/permeabilized in PBS containing 0.075% Triton X-100 and 2 mg/ml bovine serum albumin (Sigma), and incubated with primary antibody overnight at 4°C. Following washes (PBS plus 0.075% Triton X-100), cells were incubated in secondary antibody (goat anti-mouse, goat anti-rabbit Alexa 568, 633; Molecular Probes) for eight hours at 4° C and mounted using Vectashield (Vector) and #1 coverslips. Images were collected on a Zeiss 510 Meta confocal microscope (40 power oil 1.4 NA (Zeiss), pinhole equals 1.0 Airy Disc) using Carl Zeiss Imaging software. All channels were collected on PMT3. Images were imported into Adobe Photoshop for cropping and linear contrast adjustment (Mohler et al., 2004b).

I_{Ca} recordings

To determine current-voltage (I-V) relationships, cultured cardiac myocytes were stimulated at 0.5 Hz by holding at -80 mV and stepping to more-positive potentials at 10 mV intervals (T=23-25°C). For I_{Ca} facilitation, cells were stimulated by stepping from -80 to 0 mV for 150ms at 0.5 Hz. The intracellular solution contained: 120 mM CsCl, 3 mM $CaCl_2$, 10 mM tetraethylammonium

chloride, 1 mM MgATP, 1mM NaGTP, 5 mM phosphocreatine, 10 mM HEPES, 10 mM EGTA, titrated to pH 7.2 using 1 M CsOH. The cells were bathed in: 137 mM NMDG, 10 mM HEPES, 10 mM glucose, 1.8 mM $CaCl_2$, 0.5 mM $MgCl_2$, 25 mM CsCl titrated to pH 7.4 using 12.1 M HCl.

Statistics

Data are expressed as means ± S.E.M. Student's t-test was performed for paired analysis. Multiple group analyses were done using ANOVA. The null hypothesis was rejected if $p<0.05$.

CHAPTER III

L-TYPE CA^{2+} CHANNEL FACILITATION MEDIATED BY PHOSPHORYLATION OF THE BETA SUBUNIT BY CAMKII

Introduction

Intracellular calcium (Ca^{2+}) concentrations are dynamically regulated in all eukaryotic cells to permit Ca^{2+} to function as a second messenger yet prevent adverse consequences of sustained Ca^{2+} elevation. Voltage-dependent Ca^{2+} channels are major portals for Ca^{2+} entry in many cells (Mikami et al., 1989) and can regulate cell contraction (Tanabe et al., 1990), gene transcription (Dolmetsch et al., 2001), synaptic plasticity (Yasuda et al., 2003) and hormone secretion (Artalejo et al., 1994). The ion-conducting pore of voltage-gated calcium channels is formed by one of a large family of α_1 subunits, which are typically associated with auxiliary subunits and other proteins that modulate the targeting and biophysical properties of the channels (Arikkath and Campbell, 2003; Catterall, 2000). Flux through voltage-gated Ca^{2+} channels is also dynamically regulated by Ca^{2+}-dependent feedback mechanisms as well as by protein phosphorylation (Dolphin, 2003), permitting cross-talk with other second messenger signaling pathways.

The L-type voltage-gated calcium channels (LTCC) are formed from α_{1c} or α_{1d} subunits that associate with one of a family of β subunits (β_1- β_4) (Arikkath and Campbell, 2003). Ca^{2+}/calmodulin acts both directly on α_{1c} to inactivate neuronal (Zuhlke et al., 1999) and cardiac (Colecraft et al., 2002) LTCCs and indirectly via Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) to facilitate whole cell Ca^{2+} currents (I_{Ca}) in cardiac myocytes (Anderson et al., 1994; Yuan and Bers, 1994). Dodecameric CaMKII holoenzymes undergo inter-subunit Ca^{2+}/calmodulin-dependent autophosphorylation within the regulatory domain (at Thr286 in the α isoform or Thr287 in the β, γ and δ isoforms).

Autophosphorylation at Thr286/7 enhances the affinity of the kinase for calmodulin approximately 1000-fold and confers constitutive Ca^{2+}/calmodulin-independent kinase activity until dephosphorylated by a protein phosphatase. This provides a unique ability for CaMKII to integrate information conveyed by the amplitude, duration and frequency of repeated Ca^{2+} transients that cause contraction of cardiac myocytes or induce synaptic plasticity in neurons (De Koninck and Schulman, 1998). Indeed, changes in neuronal firing frequency modulate the amount of constitutive CaMKII activity (Eshete and Fields, 2001) and increasing the rate of heart contraction enhances CaMKIIδ autophosphorylation at Thr287 (Wehrens et al., 2004). Moreover, prolongation of the cardiac action potential associated with early-afterdepolarizations increases Ca^{2+}/calmodulin-independent kinase activity, consistent with enhanced Thr287 autophosphorylation of CaMKIIδ (Anderson et al., 1998). In cardiac myocytes, Ca^{2+} release from sarcoplasmic reticulum (SR) activates CaMKII to facilitate

whole cell I_{Ca} (Wu et al., 2004) and CaMKII is an essential element in excitation-contraction coupling (Wu et al., 2001a). Consistent with this role, CaMKII and the LTCC α_1 and β subunits are each localized along the Z-line of cardiac myocytes in close proximity to T-tubules (Gao et al., 1997b; O-Uchi et al., 2005; Wu et al., 1999a; Xiao et al., 1994). Moreover, CaMKII is associated with the cytosolic face of LTCCs in excised cardiac myocyte membrane patches, increasing the channel open probability (P_o) (Dzhura, 2000; Dzhura et al., 2002). Recent data suggest that CaMKII can interact with several domains in the LTCC α_1 subunit *in vitro* (Hudmon et al., 2005), but the molecular basis for localized facilitation of LTCCs by CaMKII in cardiac myocytes or any other excitable cell has not been identified.

Here we define a molecular mechanism for the actions of CaMKII that requires the β_{2a} subunit. CaMKII interacts with β_{2a} to strategically target the kinase to LTCCs, and preferentially phosphorylates β_{2a} at Thr498. Our data indicate that Thr498 phosphorylation is essential for CaMKII modulation of both single LTCCs in tsA201 cells and whole cell I_{Ca} in cardiac myocytes, thereby defining a molecular basis for CaMKII modulation of Ca^{2+} entry via LTCCs in native cells.

Results

CaMKII binds to the LTCC β_{2a} subunit

As a first step toward investigating whether LTCC β subunits play a role in CaMKII phosphorylation-mediated facilitation of cardiac LTCCs (Dzhura et al., 2000), we performed glutathione agarose co-sedimentation assays using a glutathione-S-transferase (GST) fusion protein containing the entire sequence of the rat β_{2a} subunit (GST-β_{2a}(WT)). The major cardiac and neuronal isoforms of CaMKII (CaMKIIδ and CaMKIIα, respectively) associated with GST-β_{2a}(WT), depleting kinase subunits from solution (Figure 8A). Binding required prior activation of CaMKII by autophosphorylation in the regulatory domain (Thr287 in CaMKIIδ or Thr286 in CaMKIIα). In a glutathione-plate binding assay, GST-β_{2a}(WT) bound CaMKII with apparent K_a ≈90 nM (Figure 8B), more than an order of magnitude lower than estimated levels of CaMKII in heart and brain and roughly equilivant to the levels of autonomously activated CaMKII in the heart (McNeill and Colbran, 1995; Wu et al., 1999a).

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} 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 8C). The structure of SH3/GK domains in several β subunit isoforms has been recently reported (e.g., (Van Petegem et al., 2004)) revealing insights into the mechanism for constitutive association of the α and β

subunits, but residues 410-505 were 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 8D) (Bayer et al., 2001; Strack and Colbran, 1998; Strack et al., 2000a). The binding parameters for CaMKII interaction with GST-β_{2a}(WT) were very similar to those for CaMKII interaction with GST-NR2B(1260-1339) (Figure 8A,B).

To investigate whether CaMKII associates with β_{2a} subunits in intact cells, CaMKII was co-expressed with FLAG-tagged β_{2a} proteins in HEK293 cells. Immunoprecipitations using FLAG antibodies resulted in the co-precipitation of CaMKII from cell lysates containing FLAG-β_{2a}(WT), but not from lysates that did not contain FLAG proteins (Figure 8E). 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*.

CaMKII preferentially phosphorylates Thr498 in β_{2a}

We next investigated whether CaMKII efficiently phosphorylates GST-β_{2a}(WT) in comparison to PKA, which can enhance whole cell currents (I_{Ca}) of recombinant LTCCs by phosphorylating β_{2a} at Ser478 and 479 (Bunemann et al., 1999). CaMKII phosphorylated GST-β_{2a}(WT) at a substantially faster initial rate than an equimolar concentration of PKA (Figure 9A), even though these kinases

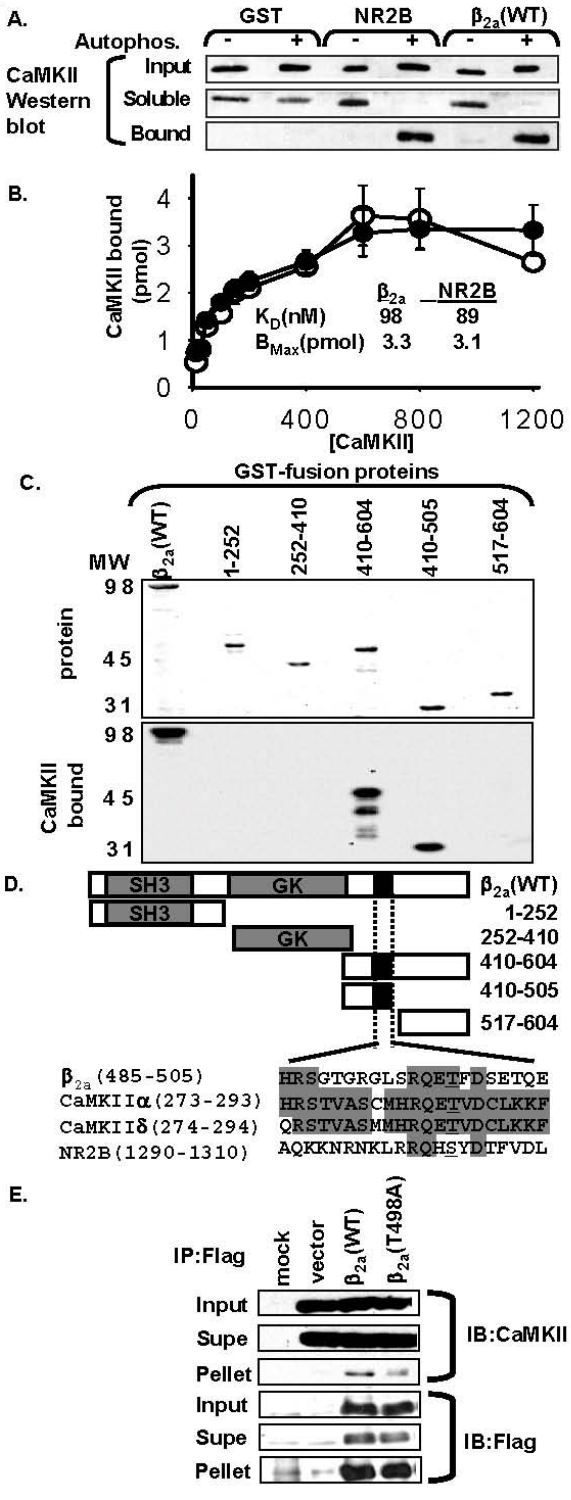

A.
GST
NR2B
β2a(WT)
Autophos.
- + - + - +
CaMKII Western blot
Input
Soluble
Bound
B.
CaMKII bound (pmol)
4
3
2
1
0
0
400
800
1200
[CaMKII]
β2a
NR2B
KD(nM) 98 89
BMax(pmol) 3.3 3.1
C.
GST-fusion proteins
MW
β2a(WT)
1-252
252-410
410-604
410-505
517-604
98
45
31
protein
CaMKII bound
D.
SH3
GK
β2a(WT)
1-252
252-410
410-604
410-505
517-604
β2a(485-505) HRSGTGRGLSRQETFDSETQE
CaMKIIα(273-293) HRSTVASCMHRQETVDCLKKF
CaMKIIδ(274-294) QRSTVASMMHRQETVDCLKKF
NR2B(1290-1310) AQKKNRNKLRRQHSYDTFVDL
E.
IP:Flag
mock
vector
β2a(WT)
β2a(T498A)
Input
Supe
Pellet
IB:CaMKII
Input
Supe
Pellet
IB:Flag

Figure 8. Binding of CaMKII to the LTCC β_{2a} subunit.
(A) Thr287 autophosphorylation-dependent binding of CaMKIIδ to GST-β_{2a}(WT) and GST-NR2B using co-sedimentation assays. The input, soluble and bound fractions were analyzed by immunoblotting for CaMKII. GST-β_{2a}(WT) displayed similar autophosphorylation-dependent binding to CaMKIIα (not shown).
(B) CaMKIIδ binds β_{2a} (●) and NR2B (○) with similar affinity in a glutathione plate binding assay. The inset table reports equilibrium binding parameters (K_d and B_{max}). Data is displayed as mean ±S.E.M.
(C) Mapping the CaMKII-binding domain to amino acids 410-505 using gel overlay assays.
(D) 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 and similarities within black and gray boxes, respectively.
(E) CaMKII co-immunoprecipitates with FLAG-β_{2a}. HEK293 cells were transiently transfected to express CaMKII with either FLAG-β_{2a}(WT) or FLAG-β_{2a}(T498A). Control cells were mock transfected or expressed CaMKII alone. Cell lysates were immunoprecipitated using anti-FLAG agarose beads and aliquots of inputs, supernatants (supe) and immune pellets (IP) were immunoblotted for CaMKII (top) and FLAG proteins (bottom). (Experiments in panel A-C were performed by Sunday Abiria)

displayed similar specific activities toward model peptide substrates (see Methods). Moreover, CaMKII phosphorylated GST-β_{2a}(WT) to a much higher final stoichiometry than PKA (Figure 9B), suggesting that CaMKII can phosphorylate multiple sites in the β_{2a} subunit. A similar CaMKII phosphorylation stoichiometry was observed using a C-terminal domain fragment of β_{2a} (residues 410-604) that contains the CaMKII-binding domain (Figure 10).

As an initial step toward identifying CaMKII phosphorylation sites(s) in β_{2a} we performed phosphoamino acid analysis on GST-β_{2a}(WT) and GST-β_{2a}(410-604) following phosphorylation by CaMKII to stoichiometries of 2.9 and 1.5 mol/mol, respectively. CaMKII phosphorylated both threonine and serine residues in each protein, whereas only serine(s) were targeted by PKA (Figure 9C). Tryptic or chymotryptic digests of CaMKII-phosphorylated GST-β_{2a}(WT) were then analyzed by tandem mass spectrometry, identifying several phosphorylation sites (Figure 11B) but providing no information about relative phosphorylation stoichiometries at each site. Among the sites identified in both tryptic and chymotryptic digests was Ser459, which was previously identified as a PKA site with no known functional role (Bunemann et al., 1999). However, mutation of all known PKA sites in β_{2a} (Ser459, Ser478 and Ser479) had no significant effect on CaMKII phosphorylation (Figure 12). Close examination of the tandem mass spectrum for a tryptic peptide containing Thr498 showed a fragmentation pattern that was consistent with Thr498 phosphorylation (Figure 11D). Thr498 lies within the CaMKII-binding domain of β_{2a}, and the surrounding amino acid sequence is similar to sequences surrounding Thr287 in CaMKIIδ,

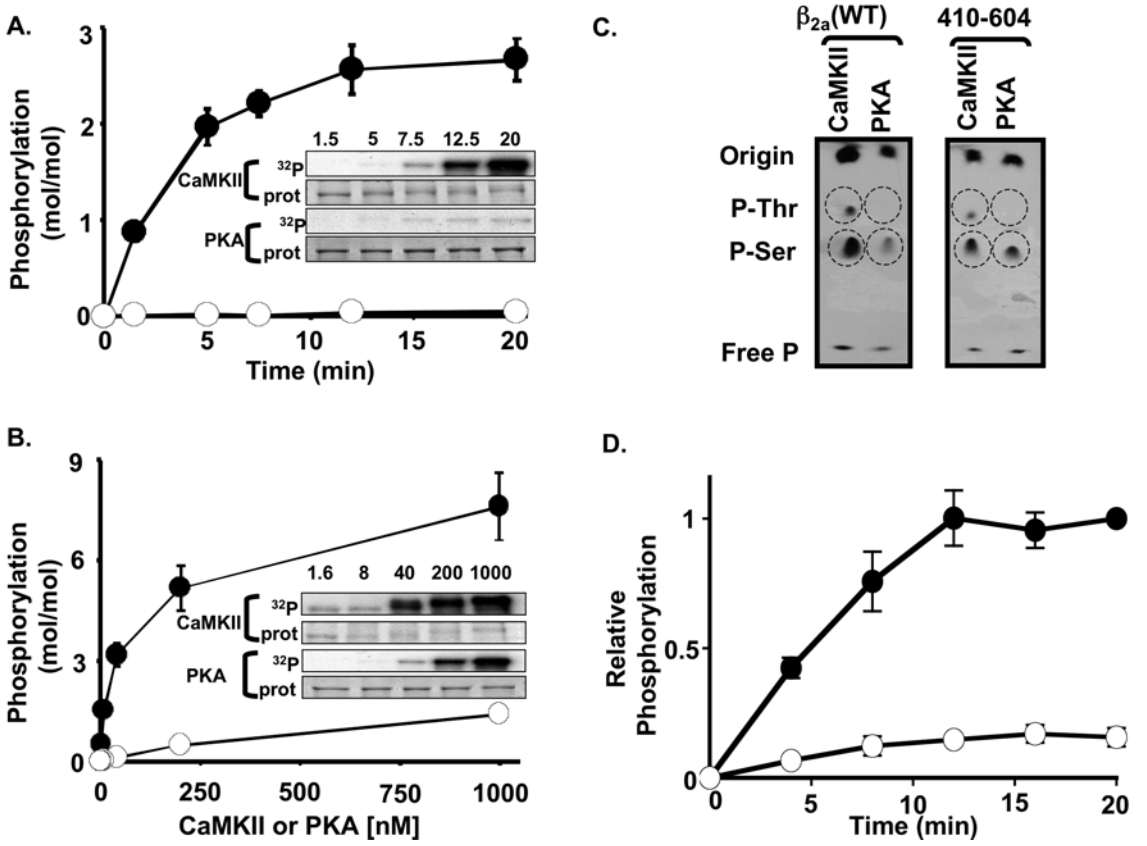


Figure 9. Thr498 in the β_{2a} subunit is a preferred CaMKII phosphorylation site.
(A) Time course for *in vitro* phosphorylation of GST-β_{2a}(WT) by CaMKII (●) and PKA (○). Aliquots were removed at the indicated times and resolved by SDS-PAGE. The Coomassie Blue stained gel and corresponding autoradiograph are shown ("prot" and "^{32}P" in the inset), along with the phosphorylation stoichiometry in the main graph ($p<0.05$ at all points). Data are displayed as mean ±S.E.M.
(B) Kinase concentration dependence of GST-β_{2a}(WT) phosphorylation. Data are displayed as in panel A ($p<0.05$ at all points). Data are displayed as mean ±S.E.M.
(C) Phosphoamino acid analysis showing that CaMKII phosphorylates threonine residues in the C-terminal domain of β_{2a}. Internal phosphoamino acid standards were detected colorimetrically and their positions are marked by dashed circles on the autoradiograph.
(D) Thr498 in β_{2a} is a preferred site for CaMKII phosphorylation. Timecourse of phosphorylation of GST-β_{2a}(WT) (●) and GST-β_{2a}(T498A) (○) using 10 nM CaMKII at 4°C normalized to GST-β_{2a}(WT) at 20 minutes ($p<0.02$ at all points). Data are displayed as mean ±S.E.M.

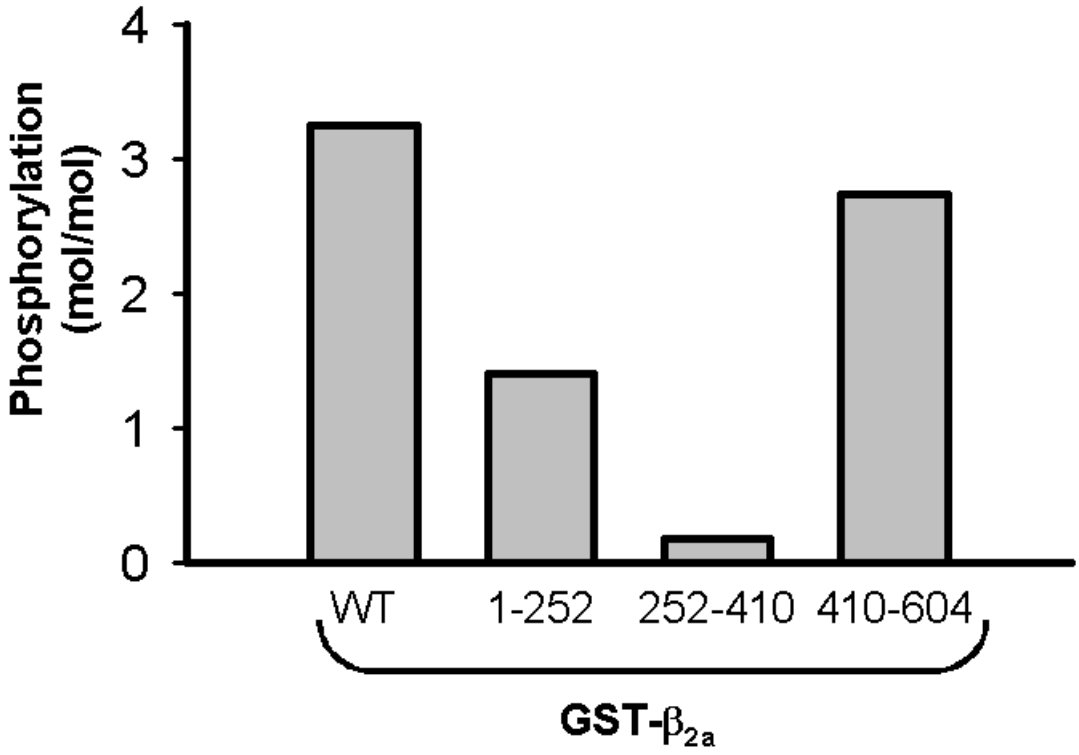


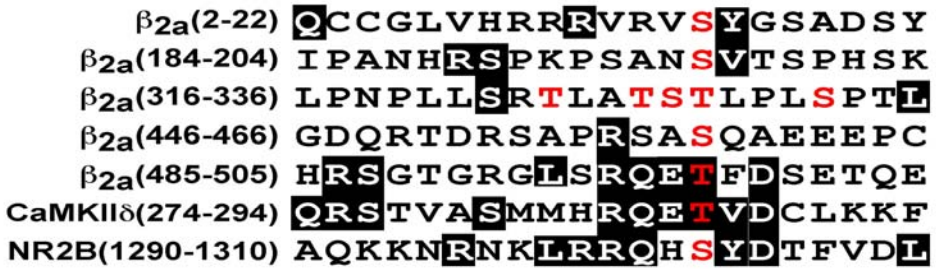
Figure 10. *In vitro* phosphorylation of β_{2a} subunit domains by CaMKII. GST-β_{2a}(WT), -β_{2a}(1-252), -β_{2a}(252-410), or -β_{2a}(410-604) (1 μM) were incubated at 30°C for 2.5 minutes with [γ-^{32}P]ATP and CaMKIIδ_2 (100 nM). Aliquots were spotted on P81 paper for scintillation counts. The graph represents average phosphorylation stoichiometries from duplicates in a single experiment that is representative of 3-4 experiments. CaMKII phosphorylates the C-terminal domain of β_{2a} (residues 410-604) to a similar stoichiometry as the full length (WT) protein, but the N-terminal domain (residues 1-252) is also phosphorylated.

A. Domain V1 C1 V2 C2 V3

B.

Peptide sequence	Domain	Phosphorylation site
VSYGSADSYTSR* / VRVSYGSADSY**	V1	Ser15
PSANSVTSPHSK* / RSPKPSANSVTSPH**	V2	Ser197
TLQLVVLDADTINHPAQLSK*	C2	Thr311
TLATSTLPLSPTLASNSQGSQGDQR*	V3	Ser/Thr 424 or 427-429 or 433
SASQAEEEPCLEPVK* / SASQAEEEPCLEPVKK* / SAPRSASQAEEEPCLEPVKK** / SAPRSASQAEEEPCLEPVKKSQ**	V3	Ser459
QETFDSETQESR *	V3	Thr498

C.



D.

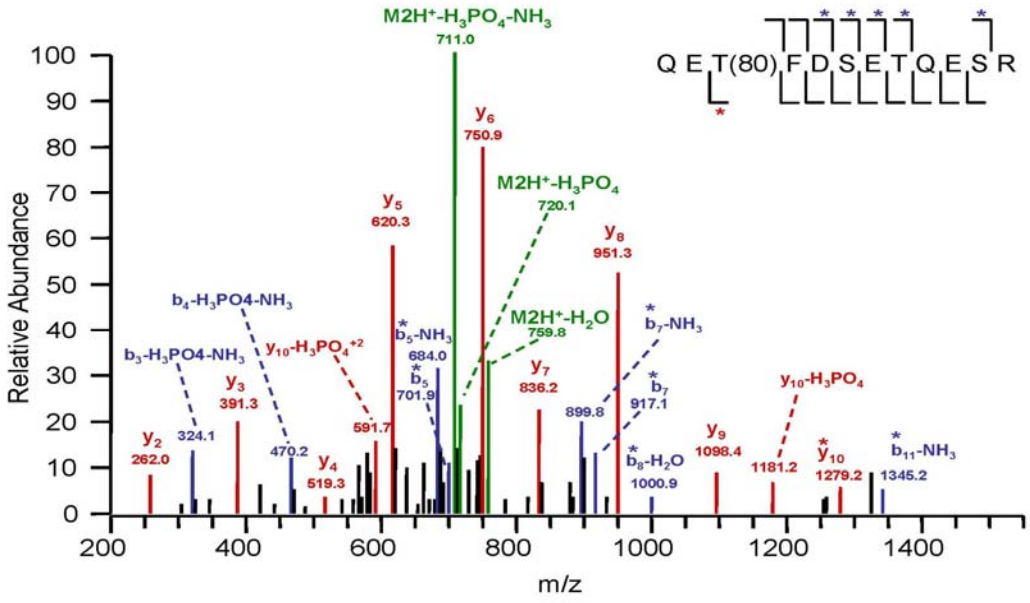


Figure 11. Tandem mass spectrometry spectrum demonstrating Thr498 phosphorylation.
GST-β_{2a}(WT) was phosphorylated to a stoichiometry of ≈3 mol/mol by CaMKII and re-purified using glutathione-agarose. The protein was digested with trypsin and then analyzed by LC-MS-MS. A tandem mass spectrum is displayed showing relative abundance of peptide fragments vs. mass/charge (mz) ratio. Y-ions are highlighted in red, b-ions in blue. An 80 dalton shift in mass from the predicted fragment value is denoted with an asterisk, indicating the presence of a phosphate. This fragmentation pattern conclusively identifies Thr498 as a phosphorylated residue. (LC-MS-MS performed by Amy Ham and the Proteomics Core)

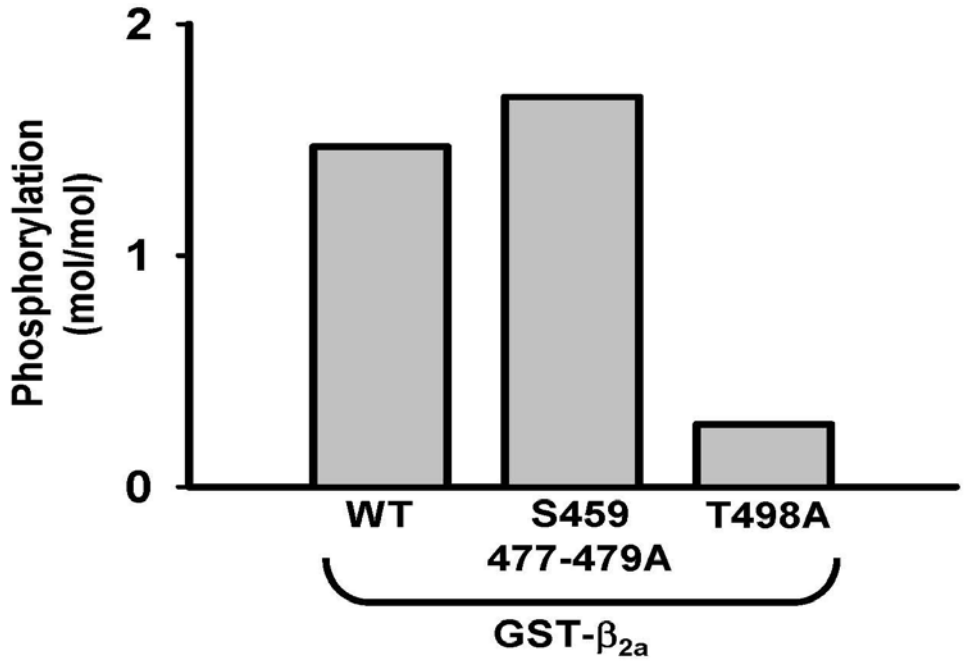


Figure 12. *In vitro* phosphorylation of β_{2a} subunit wild type, PKA site mutants and CaMKII site mutant by CaMKII.
Full length GST-β_{2a}(WT), -β_{2a}(S459, 477-479A), or -β_{2a}(T498A) (1 μM) were incubated at 30°C for 20 minutes with [γ-^{32}P]ATP and CaMKIIδ_2 (10 nM). Aliquots were spotted on P81 paper for scintillation counts. The graph represents average phosphorylation stoichiometries from duplicates in a single experiment that is representative of 2-4 experiments.

Thr286 in CaMKIIα and Ser1303 in NR2B (Figure 11C), which are all potently phosphorylated by CaMKII (Colbran, 1993; Omkumar et al., 1996). In contrast, amino acids sequences surrounding the other CaMKII phosphorylation site in GST-β_{2a} exhibited very limited similarity with these previously identified CaMKII phosphorylation sites (Figure 11C).

Mutation of Thr498 to Ala in the context of full-length β_{2a} (GST-β_{2a}(T498A)) significantly reduced the initial rate of CaMKII phosphorylation (Figure 9D) ($p<0.02$). In combination, these data show that Thr498 in the β_{2a} subunit is both a highly efficient and preferred CaMKII substrate.

CaMKII regulation of recombinant LTCCs requires Thr498 in β_{2a}

To directly test the hypothesis that the β_{2a} subunit is required for CaMKII regulation of LTCCs, α_{1c} subunits were transiently-expressed in tsA201 cells with and without β_{2a} subunits. Recordings of single LTCC activities in excised cell membrane patches confirmed previous observations (Wakamori et al., 1993; Zhang et al., 2005a) that β_{2a}(WT) significantly increases the probability of channel opening (P_o) under basal conditions (Figure 13, compare open bars). Addition of CaMKII to the cytosolic face of the membrane further increased the P_o of α_{1c}/β_{2a}(WT) channels about 2.5-fold but had no effect on channels formed from α_{1c} alone (compare black and open bars). The CaMKII-induced increase in P_o was abrogated by single point mutation of Thr498 to Ala in β_{2a} (α_{1c}/β_{2a}(T498A) channels), but this mutation did not affect basal P_o (Figure 13), or the

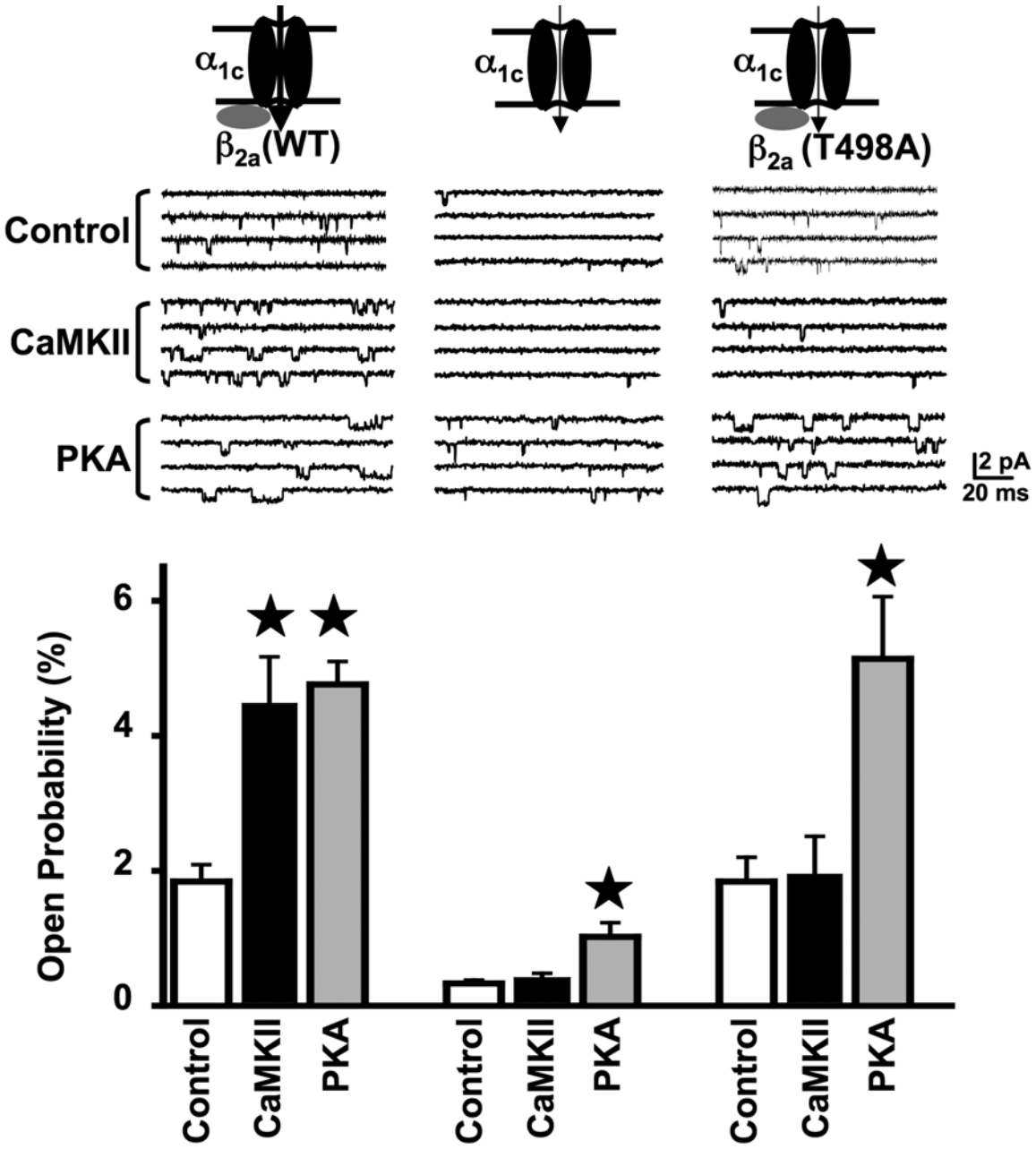


Figure 13. Thr498 in the β_{2a} subunit mediates regulation of recombinant LTCCs.

Cartoons at the top show LTCCs containing α_{1c} and β_{2a}(WT), α_{1c} alone or α_{1c} and β_{2a}(T498A) expressed in tsA201 cells. Representative sweeps of single channel activity are shown following incubation of cytosolic faces of excised membrane patches without or with constitutively active CaMKII or PKA (1 μM each). The bar graph shows cumulative open probabilities (P_o) for channels under each condition. *: $p<0.001$ compared to absence of kinase. Data are displayed as mean ±S.E.M. (Single Channel experiments performed by Igor Dzhura)

coimmunoprecipitation of CaMKII with β_{2a} subunits (Figure 8E). In contrast, PKA increased the P_o of LTCCs formed from α_{1c} alone, α_{1c}/β_{2a}(WT) or α_{1c}/β_{2a}(T498A) to a similar extent (≈2.5-fold) (Figure 13). These data show that CaMKII increases P_o by a mechanism that is distinct from that of PKA and that require Thr498 in β_{2a}, indicating that phosphorylation at this preferred site is essential for CaMKII regulation of LTCCs.

I_{Ca} facilitation in rat cardiac myocytes requires Thr498 in β_{2a}

The β subunit serves dual roles in modulating LTCC currents by acting to increase cell surface expression and to augment P_o. In order to assess the role of Thr498 in the β_{2a} subunit in native cells, FLAG-β_{2a}(WT) or FLAG-β_{2a}(T498A) proteins were expressed downstream of GFP and an internal ribosome entry site in freshly isolated adult rat cardiac myocytes using recombinant lentivirus. Cells were initially analyzed by immunofluorescence using anti-FLAG and anti-CaMKII antibodies approximately 15 hours after viral transduction. Confocal imaging revealed that both exogenous FLAG-β_{2a} subunits (WT or T498A) as well as the endogenous CaMKII are present in punctae along the cardiac myocyte Z-line, overlapping with α-actinin fluorescence (Figure 14), and consistent with their normal localization to T-tubule membranes (Gao et al., 1997b). Endogenous CaMKII was similarly localized along the Z-line in non-transduced cells and in cells transduced with control lentivirus. Strikingly, many of the CaMKII punctae co-localized with FLAG-β_{2a}(WT) or FLAG-β_{2a}(T498A) punctae, although some CaMKII displayed a unique localization (Figure 15A). These data show that

CaMKII and the FLAG-β_{2a} subunits are present in the same subcellular compartment in cardiac myocytes.

Whole cell patch clamp analyses of cardiac myocytes transduced with control, FLAG-β_{2a}(WT) or FLAG-β_{2a}(T498A) lentivirus demonstrated that the general form of the current-voltage (I-V) relationship was similar. However, the maximum I_{Ca} density was increased by over-expression of FLAG-β_{2a}(WT) or FLAG-β_{2a}(T498A) (-8.4±0.51 pA/pF and -8.7±0.45 pA/pF, respectively) compared to control cells (-6.8±0.51 pA/pF) (Figure 15B). The similar modest increases in peak I_{Ca} 15-24 hours after lentiviral expression of FLAG-β_{2a}(WT) and FLAG-β_{2a}(T498A) are considerably less than reported in previous studies that overexpressed β_{2a} (Chen et al., 2005; Colecraft et al., 2002; Wei et al., 2000). Since β subunits are thought to be rate-limiting for expression of functional LTCCs (Wei et al., 2000), this discrepancy likely reflects the relative expression levels of exogenous β subunits due to differences in viral technology used for protein expression and/or the time at which analyses were performed. Together, these data show that mutation of Thr498 to Ala does not affect the I-V relationship, disrupt the chaperone activity of β_{2a} subunits, or affect the normal pattern of CaMKII localization in adult cardiac myocytes.

CaMKII-dependent I_{Ca} facilitation is a unique physiological readout of CaMKII action in cardiac myocytes that has not been demonstrated in heterologous cells. Therefore, we investigated the role of Thr498 in β_{2a} in CaMKII-dependent facilitation of I_{Ca} in cardiac myocytes (Dzhura et al., 2000; Wu et al., 1999a). Facilitation was preserved in untransduced myocytes cultured for

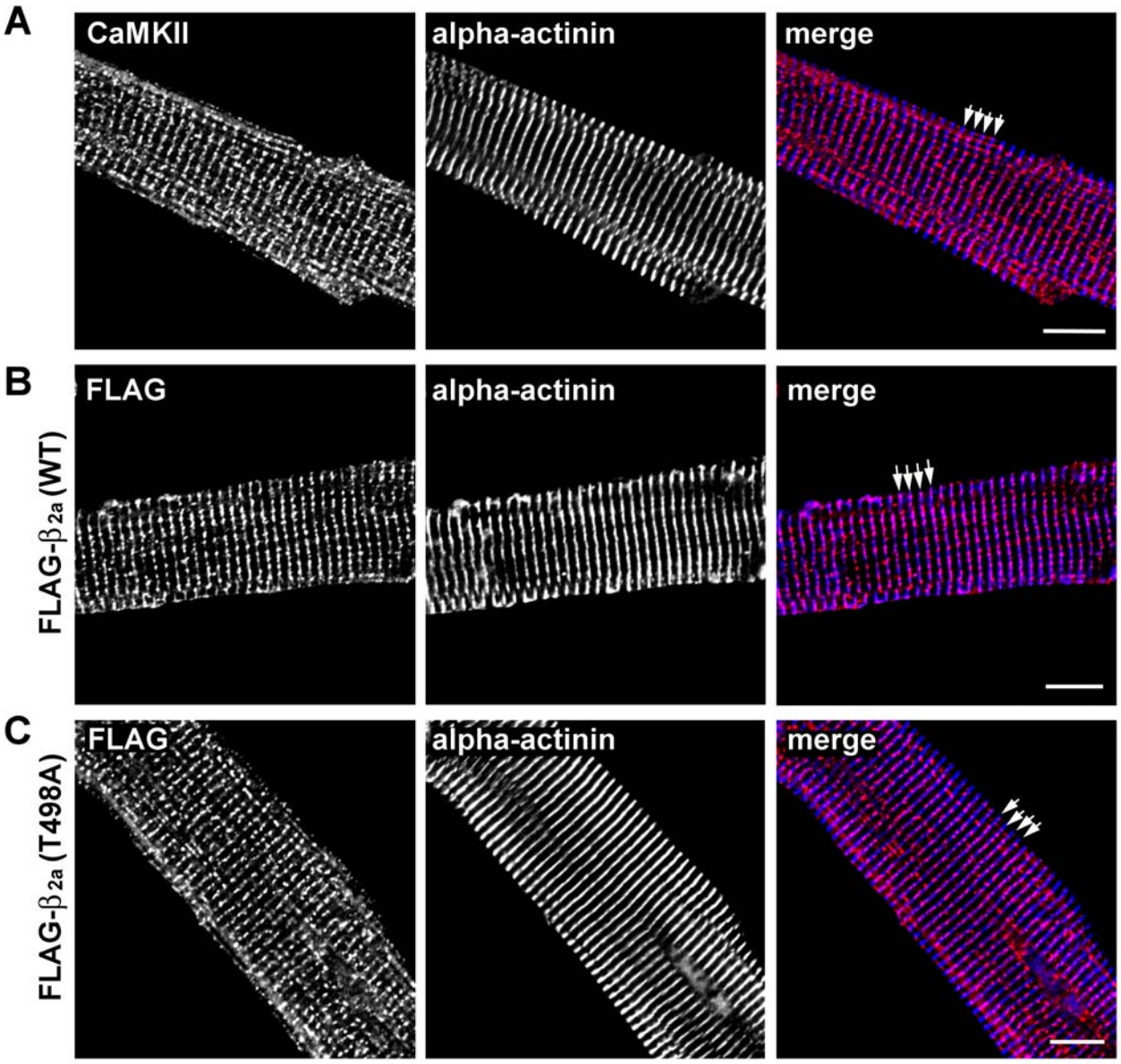


Figure 14. Immunolocalization of endogenous CaMKII and exogenously expressed FLAG-β_{2a} WT and T498A lentiviral constructs in adult rat cardiac myocytes.
Note that both CaMKII and FLAG β subunits (in red) are primarily localized over the Z-line with α-actinin (blue). Pink represents the overlapping signal between red and blue stains. Scale bars equal ten microns. (Images by Peter Mohler and myself)

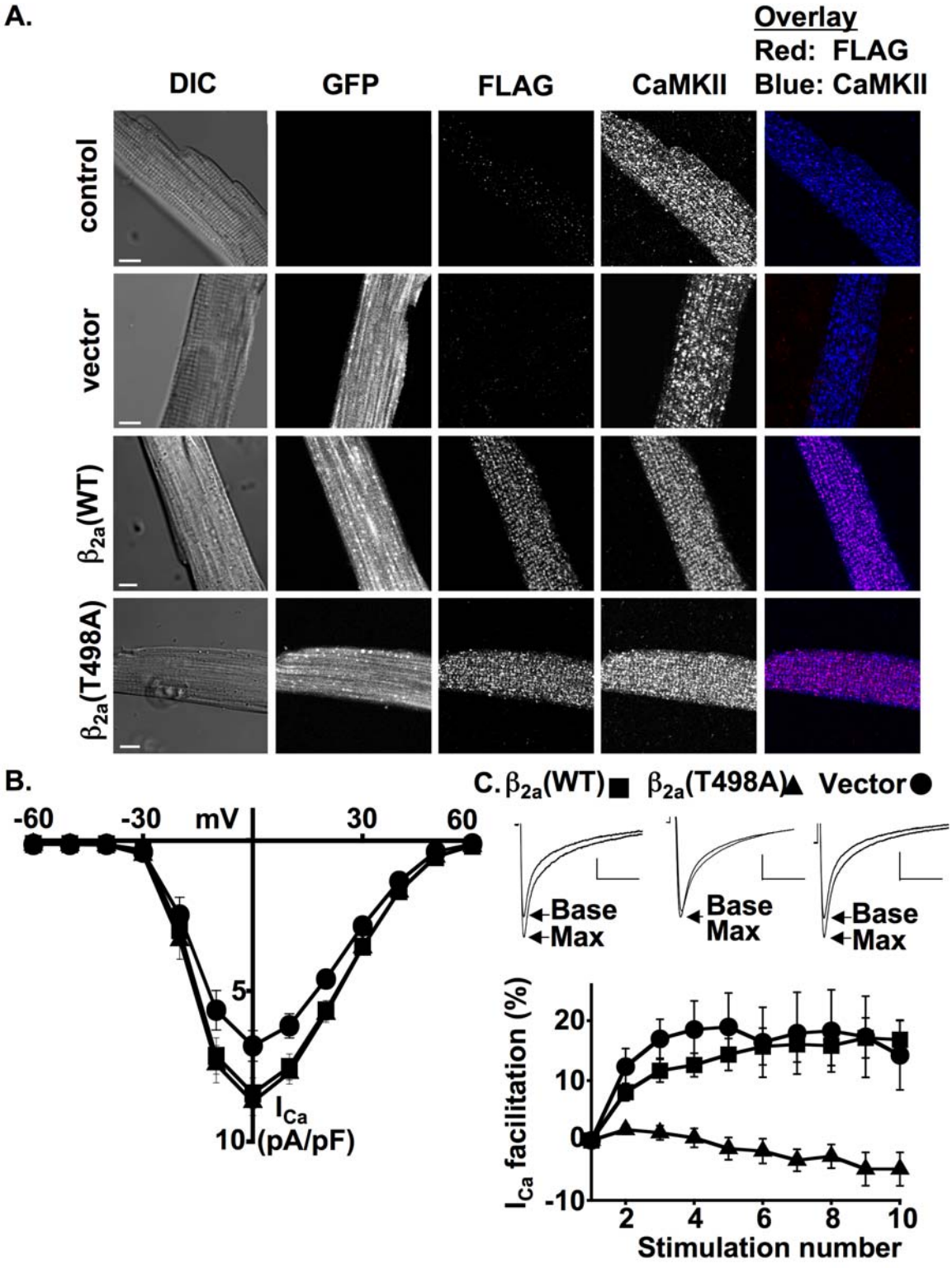

A.
Overlay
Red: FLAG
Blue: CaMKII
DIC
GFP
FLAG
CaMKII
control
vector
β2a(WT)
β2a(T498A)
B.
-60
-30
mV
30
60
5
10
ICa
(pA/pF)
C. β2a(WT)■ β2a(T498A)▲ Vector●
Base
Max
Base
Max
Base
Max
ICa facilitation (%)
20
10
0
-10
2
4
6
8
10
Stimulation number

Figure 15. I_{Ca} facilitation in rat cardiac myocytes requires Thr498 in β_{2a}.
(A) Confocal images of GFP fluorescence and immunolocalization of FLAG-β_{2a} proteins and CaMKII to T-tubules in non-transduced adult rat cardiac myocytes cells (control) and in cells transduced with control lentivirus (vector: expresses GFP alone), FLAG-β_{2a}(WT) lentivirus or FLAG-β_{2a}(T498A) lentivirus.
(B) Current-voltage (I-V) relationships for whole-cell Ca^{2+} currents (I_{Ca}) were unaltered, but peak I_{Ca} was modestly increased in cardiac myocytes transduced with FLAG-β_{2a}(WT) lentivirus (■: n=12) or FLAG-β_{2a}(T498A) lentivirus (▲: n=14), relative to control lentivirus (●: n=12). Data is displayed as mean ±S.E.M.
(C) Facilitation of I_{Ca}. Repetitive depolarization protocols (0.5 Hz) revealed normal facilitation in cardiac myocytes transduced with control (●: n=7) or FLAG-β_{2a}(WT) (■: n=7) lentivirus, but not in cells transduced with FLAG-β_{2a}(T498A) lentivirus (▲: n=10). Representative current traces are shown above with horizontal and vertical scale bars representing 50 ms and 2 pA/pF, respectively. Data are displayed as mean ±S.E.M. (Panel A images by Peter Mohler and myself, panel B by Yuejin Wu and myself)

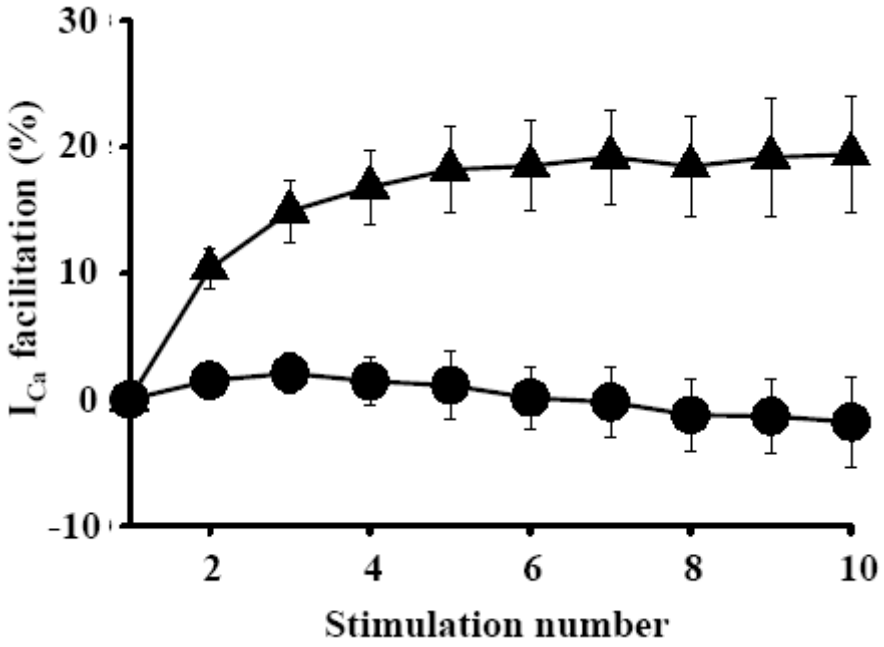


Figure 16. I_{Ca} facilitation in cultured rat cardiac myocytes.
Whole-cell voltage clamp analysis of adult rat cardiac myocytes cultured for 15-24 hours. Cardiac myocytes were repetitively depolarized from -80mV to 0mV (150 ms each) at 0.5 Hz under control conditions (▲) (n=24) or with 20 μM AC3-I CaMKII inhibitor peptide (●) (n=4) in the pipet solution. The graph shows I_{Ca} for each stimulation, expressed as the percentage change from I_{Ca} observed upon the first stimulation. As previously observed in freshly isolated murine (Dzhura et al., 2000) and rabbit (Wu et al., 2001a) cells, cultured adult rat cardiac myocytes exhibit CaMKII-dependent I_{Ca} facilitation because AC3-I prevents any changes.

15-24 hours (19.4±0.05%), as well as in cells transduced with control lentivirus (18.9±0.06%) (Figure 15C and Figure 16). Moreover, intracellular perfusion with a CaMKII inhibitory peptide (AC3-I) blocked I_{Ca} facilitation, demonstrating that CaMKII activity is required for facilitation in cultured cardiac myocytes (Figure 16). Normal I_{Ca} facilitation was observed in cardiac myocytes expressing FLAG-β_{2a}(WT) (17.1±0.03%), but no facilitation was detected in cells expressing FLAG-β_{2a}(T498A) (Figure 15C). Thus, targeted FLAG-β_{2a}(T498A) over-expression ablates I_{Ca} facilitation, demonstrating that CaMKII-dependent I_{Ca} facilitation requires β_{2a} phosphorylation at Thr498 in cardiac myocytes.

Discussion

The present studies define a molecular mechanism for CaMKII-mediated facilitation of Ca^{2+} influx via LTCCs in adult cardiac myocytes. PKA is known to enhance I_{Ca} by phosphorylating the α_{1c} subunit at Ser1928 (Gao et al., 1997b) and/or the β_{2a} subunit at Ser478 and Ser479 (Bunemann et al., 1999). However, recent work has challenged the functional relevance of PKA phosphorylation at these sites (Ganesan et al., 2006), and we show here that β subunits are not necessary for PKA-mediated increases in the P_o of recombinant channels (Figure 13B). Optimal PKA regulation of LTCCs requires targeting of PKA to the channel by the scaffolding proteins AKAP15 (Hulme et al., 2003) and/or AKAP 79 (Gao et al., 1997b). In contrast, autophosphorylated (activated) CaMKII directly binds to β_{2a} with an apparent K_D at least 10-fold lower than physiological concentrations

of CaMKII (Figure 8), and CaMKII colocalizes with β_{2a} on T-tubules at the Z-line of cardiac myocytes (Figure 15A). The β_{2a} subunit is required for the CaMKII-stimulated increase in P_O of recombinant LTCCs and we show that a preferred CaMKII phosphorylation site (Thr498) within the CaMKII-binding domain is required for the increase in P_O (Figures 10, 14B). Most significantly, expression of the β_{2a} subunit lacking Thr498 abrogates I_{Ca} facilitation in adult cardiac myocytes (Figure 15C), presumably because the recombinant mutated protein replaces endogenous β subunits in active LTCC complexes.

Expression of FLAG-β_{2a} had no significant effect on the maximum extent of LTCC facilitation (Fig. 16C), suggesting that the endogenous β subunit(s) are functionally analogous to the β_{2a} subunit in terms of CaMKII-dependent facilitation. The β_2 regulatory subunits are thought to predominate in cardiac myocytes (Colecraft et al., 2002; Foell et al., 2004), and residues 486-500 are present in all of the known β_2 subunit splice variants (Colecraft et al., 2002; Foell et al., 2004). Consequently, our findings strongly suggest that phosphorylation of β_2 subunits is required for Ca^{2+}-dependent feedback facilitation of LTCC by CaMKII in native cardiac myocytes. The β_{1b} and β_{3b} subunits that also are present in heart also contain amino acid sequences similar to that surrounding Thr498, but their relative expression levels in the cardiac myocyte and their capacity for influencing α_1 subunit gating behavior are incompletely understood. Although additional studies will be needed to determine whether other β subunit isoforms can play a similar role to the β_{2a} subunit, the sequence homology between β subunit isoforms and their broad expression profiles suggest that β

subunit mediated regulation of LTCCs by CaMKII will be important across a wide range of cell types.

CaMKII was recently reported to bind to the α_{1c} C-terminus, close to multiple calmodulin-binding motifs, as well as to other intracellular domains in the α_{1c} subunit (Hudmon et al., 2005). These authors expressed an α_{1C} subunit with a mutated calmodulin-binding motif in oocytes and showed that Ca^{2+}-dependent facilitation was disrupted by additional mutations that blocked CaMKII binding to the α_{1c} C-terminal domain *in vitro*. However, these additional mutations did not block association of CaMKII with the intact LTCC complex, consistent with our results identifying the β subunit as a CaMKII binding partner. The relationship between facilitation of these mutated recombinant channels and CaMKII-dependent facilitation of native LTCCs in cardiac myocytes is unclear, particularly because there is no evidence that CaMKII activity is required for facilitation in oocytes and no specific phosphorylation sites were identified (Hudmon et al., 2005). It is interesting to note that calmodulin-binding domains in the α_{1c} C-terminus can act as ligands to facilitate LTCCs and I_{Ca} by a biophysical mechanism that is indistinguishable from CaMKII-dependent I_{Ca} facilitation (Dzhura et al., 2003; Wu et al., 2001b). In addition, we recently reported that the β_{2a} subunit interacts with the α_{1c} C-terminus in a calmodulin-sensitive manner (Zhang et al., 2005a). Thus, the present studies show that Thr498 phosphorylation of the β_{2a} subunit is critical for CaMKII-dependent I_{Ca} facilitation in native cardiac myocytes, but they cannot exclude downstream regulatory role(s) for other mechanisms that have been previously defined in heterologous

cells, including CaMKII binding to and/or phosphorylation of other proteins in the LTCC complex, including the α_1 subunit (Erxleben et al., 2006; Hudmon et al., 2005).

CaMKII activity is increased in several forms of heart disease (Zhang and Brown, 2004) and transgenic overexpression of CaMKII is sufficient to cause cardiomyopathy (Zhang et al., 2002; Zhang et al., 2003), which is marked by increased LTCC P_o and disordered Ca^{2+} homeostasis (Maier et al., 2003; Schroder et al., 1998). Moreover, CaMKII inhibition reduces cardiomyopathy after myocardial infarction and β adrenergic receptor activation (Zhang et al., 2005b). Phosphorylation of the ryanodine receptor at CaMKII sites is enhanced in cardiomyopathy (Ai et al., 2005; O-Uchi et al., 2005; Zhang et al., 2003), and rate-dependent CaMKII-mediated phosphorylation of the ryanodine receptor is defective in a heart failure model (Wehrens et al., 2004). The present findings suggest that β_{2a} may be an additional target for CaMKII in heart disease.

This work provides critical mechanistic insight to a growing body of evidence implicating CaMKII as a universal coordinator of Ca^{2+} homeostasis. The autoregulatory properties of CaMKII are uniquely adapted to this role (De Koninck and Schulman, 1998). In cardiac myocytes, CaMKII also modulates SR Ca^{2+} release via the ryanodine receptor and SR Ca^{2+} uptake via phospholamban and the SR Ca^{2+}-ATPase (Ji et al., 2003; Ji et al., 2006; O-Uchi et al., 2005), but mechanisms for targeting CaMKII to these proteins are not well defined. While the present study focused on feedback regulation of cardiac LTCCs by CaMKII, similar mechanisms are likely to operate in many other tissues because both

CaMKII and the β_{2a} subunit are widely expressed, particularly in the brain (Bayer et al., 1999; Dolphin, 2003). Moreover, β_{2a} subunits modulate several voltage-gated calcium channel α_1 subunits (Dolphin, 2003), and these channels also exhibit Ca^{2+}/CaM-dependent facilitation (Lee et al., 2000). Thus, the CaMKII-β_{2a} mechanism described here for facilitation of cardiac LTCCs may be a critical component in the modulation of cardiac, neuronal and endocrine signaling pathways implicated in excitation-contraction coupling (Tanabe et al., 1990), transcription (Dolmetsch et al., 2001), exocytosis (Artalejo et al., 1994), action potential physiology (Wu et al., 2004), and synaptic plasticity (Grover and Teyler, 1990).

CHAPTER IV

A REGULATED INTERACTION BETWEEN CAMKII AND SELECT VGCC BETA SUBUNITS

Introduction

Voltage-gated Ca^{2+} channels are multimeric protein complexes consisting of a pore forming α_1 subunit that is usually associated with auxiliary β, $\alpha_2\delta$ and γ subunits (Arikkath and Campbell, 2003). They are defined by the biophysical and pharmacological properties of the α_1 subunit (Catterall, 2000; Hille, 2001). The high-voltage activated Ca^{2+} channels (L, N, P, Q, and R type) interact with and are regulated by the cytosolic β subunits. Four genes encoding β isoforms have been identified ($\beta_{1\text{-}4}$) each having multiple mRNA splice variants (Dolphin, 2003; Perez-Reyes et al., 1992). The β isoforms function to regulate the biophysical properties of the VGCC complex (Dolphin 2003) and act as molecular chaperones enhancing surface expression of the complex (Bichet et al., 2000).

Regulating the subcellular localization of a signaling molecule enhances the specificity of the response (Pawson and Nash, 2003). A well defined example is the regulation of PKA localization and signaling by AKAPs (Smith et al., 2006). The multifunctional Ca^{2+}/CaM dependent protein kinase II (CaMKII) regulates diverse cellular functions in response to changes in intracellular Ca^{2+}

concentrations and achieves signal specificity by regulated interaction with its target protein (Colbran, 2004; Grueter et al., 2006; Strack et al., 2000a). CaMKII is activated by Ca^{2+} bound CaM directly interacting with the regulatory domain within CaMKII (Meyer et al., 1992). Upon activation of two adjacent subunits within the dodecameric holoenzyme CaMKII can trans-autophosphorylate at Thr287 resulting in constitutive activity. It is through this structure/function relationship that CaMKII is thought to act as a molecular integrator of Ca^{2+} transients. Depending on the frequency, duration and amplitude of Ca^{2+} transients, CaMKII is autophosphorylated and remains active in the absence of Ca^{2+} (De Koninck and Schulman, 1998). Thus a direct interaction with the target protein would help ensure an accurate and timely response to stimulation such as increased intracellular Ca^{2+} in the microdomain of high voltage-gated Ca^{2+} channels (VGCC).

CaMKII modulates VGCC's Ca^{2+} current (I_{Ca}) in many cell systems including neurons and cardiac myocytes. CaMKII activation via L-type Ca^{2+} channels leads to a depression of R-type Ca^{2+} channels in neuronal spines (Yasuda et al., 2003). CaMKII directly interacts with and regulates T-type Ca^{2+} channels at the II-III linker on the α_1 subunit (Wolfe et al., 2003; Yao et al., 2006). Recent work in heterologous cells demonstrates that the α_1 subunit is a CaMKII substrate and can mediate both Ca^{2+}- and voltage-dependent facilitation in normal and disease states (Erxleben et al., 2006; Hudmon et al., 2005; Lee et al., 2006). We have recently defined a molecular mechanism for CaMKII regulation of LTCC Ca^{2+}-dependent facilitation mediated by the β_{2a} subunit (Grueter et al.,

2006). We also demonstrated that β_{2a} can act as a CaMKII anchoring protein (CaMKAP) localizing CaMKII to the LTCC complex potentially enhancing both the Ca^{2+} and voltage-dependent regulation of LTCC by CaMKII. In the current study we show that CaMKII interacts selectively with β subunit isoforms and define the CaMKII binding domain. Thr498 is a critical residue involved in CaMKII mediated regulation of LTCC (Grueter et al., 2006), and lies within the binding domain. Phosphorylation of Thr498 specifically regulates CaMKII interactions with the β subunit.

Results

CaMKII efficiently phosphorylates β_{1-4} subunits

CaMKII regulation of LTCC can occur via phosphorylation of β_{2a} Thr498, although many other sites can be phosphorylated (Grueter et al., 2006). In order to begin to analyze the potential contribution of the CaMKII phosphorylation sites in the other β isoforms, we measured the initial rates of phosphorylation (see methods) (Figure 17a). The rates of phosphorylation of β_{1b} and β_{2a} were indistinguishable but β_3 and β_4 were phosphorylated at slower rates. Aligned amino acid sequences of the domain surrounding β_{2a} Thr498 with the other three β subunit isoforms shows similarities between all four isoforms(Figure 18a). However, variations at specific amino acid residues that are likely to affect CaMKII phosphorylation were evident. The CaMKII consensus phosphorylation motif LXRXXS/T is present in both β_{2a} and β_{1b} and there is substantial amino acid

sequence identity outside this motif. The β_3 and β_4 subunits are missing key residues from the consensus phosphorylation motif and exhibit significantly less overall identity.

We next investigated the extent of CaMKII phosphorylation of the β isoforms *in vitro* (Figure 17b). CaMKII phosphorylates all β isoforms with a similar concentration dependence but the maximal phosphorylation varied somewhat between the β isoforms ($\beta_{2a,}$ β_{1b}, β_3 and β_4: 13.1±1.3, 10.8±0.4, 7.4±0.5 and 9.6±1.7 moles of phosphate/mole β respectively). Together these data show that all β subunit isoforms are CaMKII substrates but that initial phosphorylation may vary.

Select VGCC β subunits interact with CaMKII in vitro

Thr498 lies within the CaMKII-binding domain in β_{2a}. Based on the amino acid sequence comparison we hypothesized that β_{1b} would interact with CaMKII in a similar manner as β_{2a} and neither β_3 nor β_4 would bind CaMKII. To test the hypothesis we performed GST immobilization assays in glutathione coated 96-well plates using purified GSTβ subunits incubated with purified autophosphorylated CaMKII (Figure 18b). CaMKII interacts with β_{1b} and β_{2a} but not with β_3 or β_4 (data not shown). The data was fit using nonlinear regression with the calculated relative K_d for β_{1b} and β_{2a} at about 36 and 121nM, respectively, well within the estimated levels of CaMKII in neurons and cardiac myocytes (McNeill and Colbran, 1995; Wu et al., 1999a).

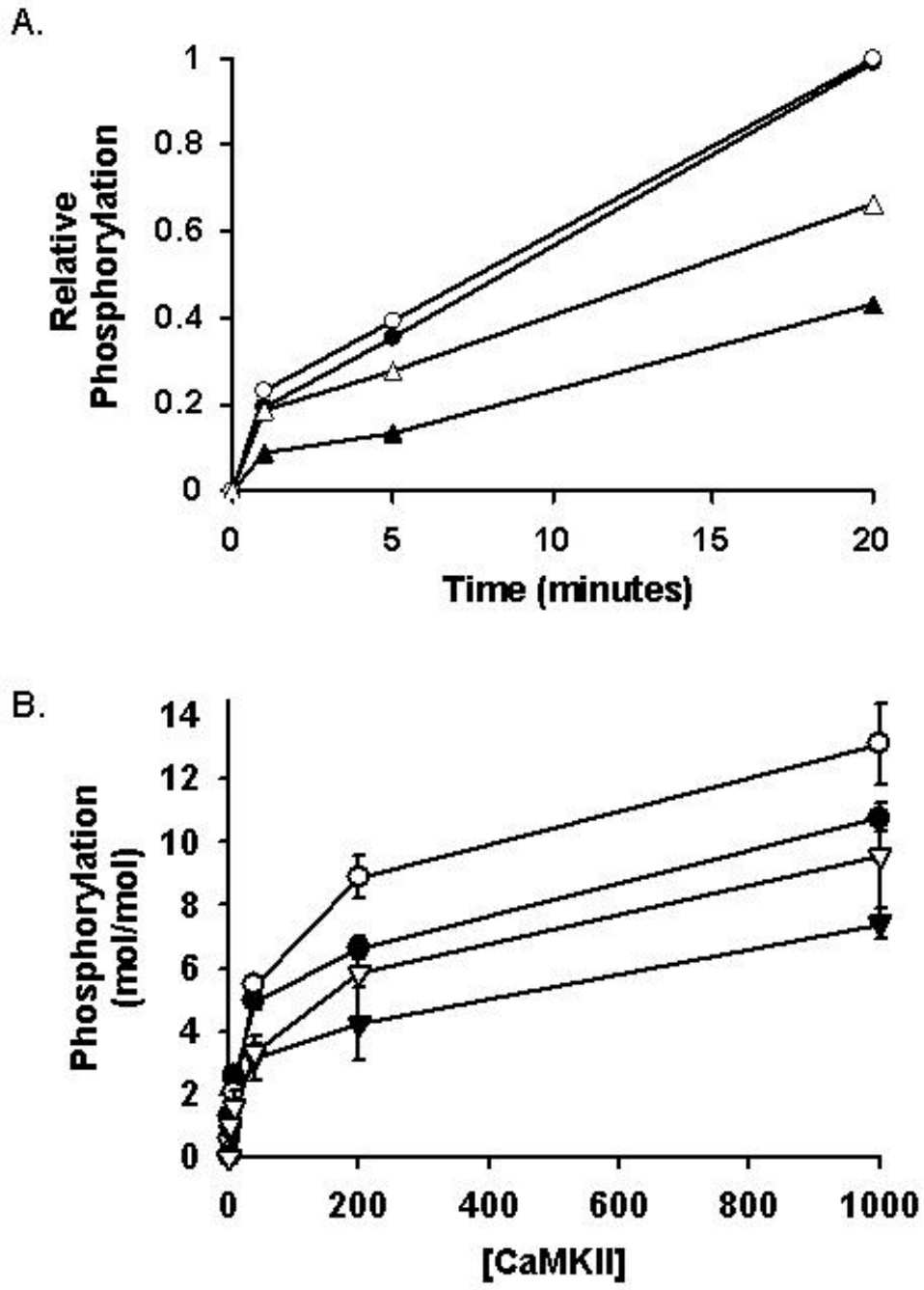


Figure 17. The β subunit isoforms are excellent CaMKII substrates *in vitro*. A. Initial rate of β isoform phosphorylation by CaMKIIδ2. (n=2). B. GSTβ isoform phosphorylation by increasing concentrations of CaMKIIδ2. (β_{2a}○, β_{1b}●, β_3▼, β_4▽). (error bars are ±S.E.M.) (n=3)

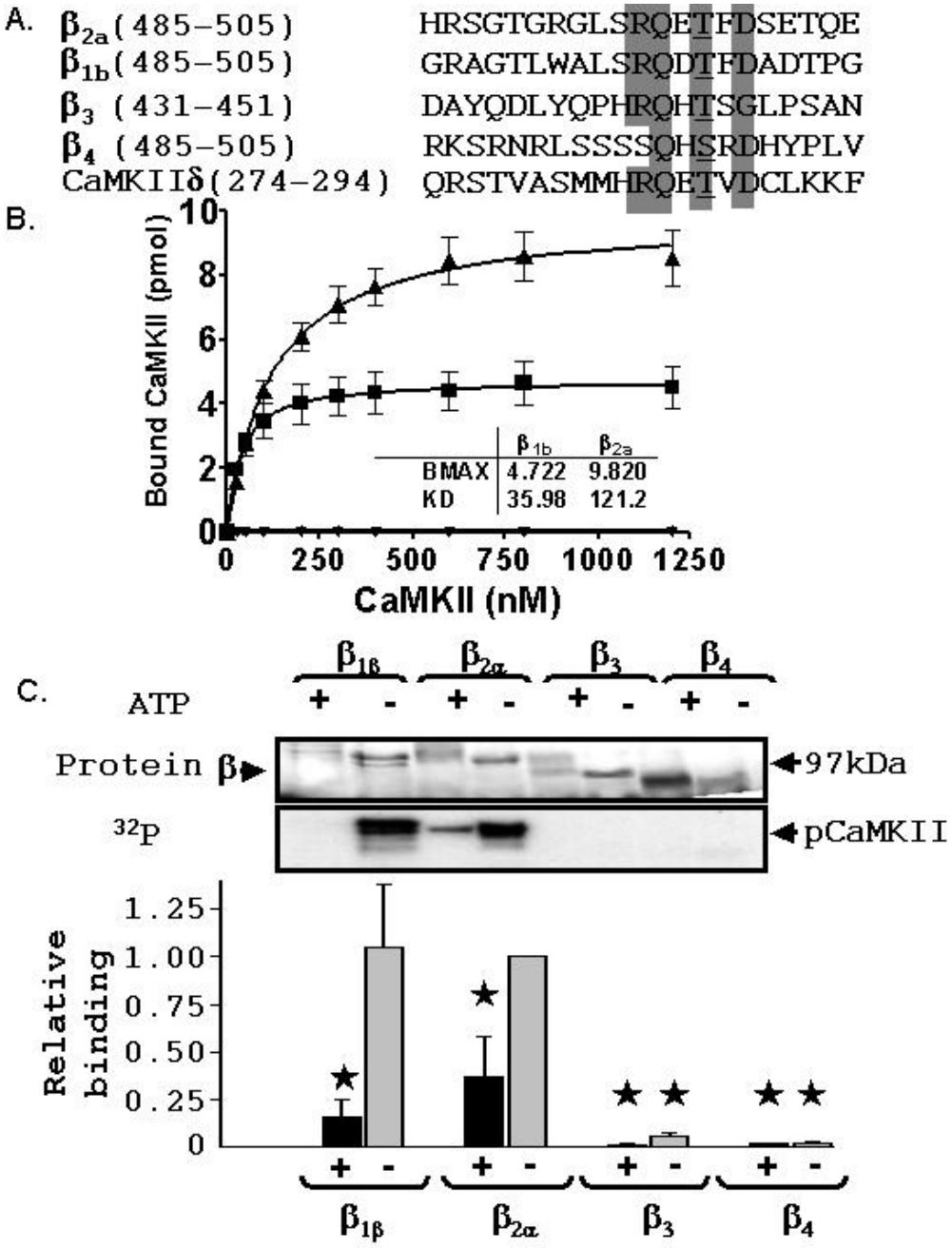

A.
β2a(485–505) HRSGTGRGLSRQETFDSETQE
β1b(485–505) GRAGTLWALSRQDTFDADTPG
β3 (431–451) DAYQDLYQPHRQHTSGLPSAN
β4 (485–505) RKSRNRLSSSSQHSRDHYPLV
CaMKIIδ(274–294) QRSTVASMMHRQETVDCLKKF
B.
Bound CaMKII (pmol)
CaMKII (nM)
β1b β2a
BMAX 4.722 9.820
KD 35.98 121.2
C.
ATP
Protein β
97kDa
32P
pCaMKII
Relative binding

Figure 18. CaMKII association with VGCC β subunit isoforms.
A. Sequence alignment of a homologous domain within $\beta_{(1-4)}$ and the CaMKIIδ autoregulatory domain surround Thr287 (underlined). Identical residues are boxed. B. Auto-phosphorylated CaMKIIδ2 interacts with β_{2a} and β_{1b} *in vitro* by glutathione plate binding assay. C. Preincubation of GST-β isoforms with CaMKIIδ2 in the presence or absence of ATP. Protein staining is depicted in the top panel. The middle panel is a representative autoradiograph from the ^{32}P CaMKIIδ2 overlay assay. The graph represents the quantitative results from three experiments. (error bars are ±S.E.M.) (Experiments in panel B were performed by Sunday Abiria)

β_{1b} and β_{2a} interact with autophosphorylated CaMKII and contain an excellent phosphorylation site within the CaMKII-binding domain. To test whether β subunit phosphorylation regulates CaMKII binding we pre-incubated CaMKII with GSTβ with or without ATP. We then performed a CaMKII overlay assay to determine CaMKII interaction (Figure 18c). Preincubation with ATP and CaMKII resulted in a gel shift in the Coomassie Blue stained protein band for the β subunit when compared to non-phosphorylated lanes, consistent with phosphate incorporation (Figure 18c protein). Consistent with Figure 18a and b, CaMKII binds non phosphorylated β_{1b} and β_{2a} but not β_3 or β_4. However, pre-phosphorylation of the β subunits significantly reduced CaMKII binding by about 80% and 70% for β_{1b} and β_{2a}, respectively. The graph represents cumulative results from three independent binding experiments. These data suggest a mechanism for autoregulation of CaMKII interaction with β_{1b} and β_{2a}.

To dissect the mechanism for regulation of CaMKII binding to β_{2a} we mutated Thr498 to Glu (to mimic phosphorylation) or Ala (to remove the phosphorylation site). The Thr498Glu mutant significantly reduced CaMKII binding by >90% in glutathione plate binding assays, whereas the Thr498Ala mutation had no significant effect.

Similarly, the Thr498Glu mutation reduced binding by about 80% in the CaMKII overlay assays but the Thr498Ala mutation had no significant effect. Both the mutated β_{2a} proteins were significantly phosphorylated by CaMKII, as evidenced by a shift in electrophoretic mobility on SDS-PAGE (and see below). However, pre-phosphorylation by CaMKII had no significant effect on CaMKII

binding to either Thr498Ala or Thr498Glu mutated β_{2a}. Together these results suggest that CaMKII interaction with β_{2a} is specifically regulated by phosphorylation of β_{2a} Thr498.

Disruption of CaMKII binding does not affect phosphorylation of β_{2a}

In order to further investigate the relationship between CaMKII binding to β_{2a} and the phosphorylation of β_{2a} we created a mutated β_{2a} protein based on previous work on another CaMKAP, the NR2B subunit of the neuronal ligand-gated Ca^{2+} channel the NMDA receptor. That work showed that mutation of the –5 site from Leu to Ala significantly reduced interaction between CaMKII and NR2B (Strack et al., 2000a). Mutating the homologous site in β_{2a}, Leu493 to Ala also significantly reduced CaMKII interaction by >90%. The Leu493Ala mutant had significantly reduced binding in the overlay assay to about 10%. Phosphorylation of the mutant protein appeared to further reduce its interaction, suggesting that Thr498 could still be phosphorylated.

The Thr498Ala and Thr498Glu mutations substantially reduced the initial rate of phosphorylation consistent with the fact that Thr498 is the initial site for CaMKII phosphorylation. The initial rate of phosphorylation of Leu493Ala by CaMKII at 4ºC was indistinguishable from the phosphorylation of wild type β_{2a} (Figure 20a). However, it seemed possible that the Leu493Ala mutation might alter the Thr498 specificity exhibited by CaMKII under these conditions. To address this question, we exploited the similarities in protein sequences

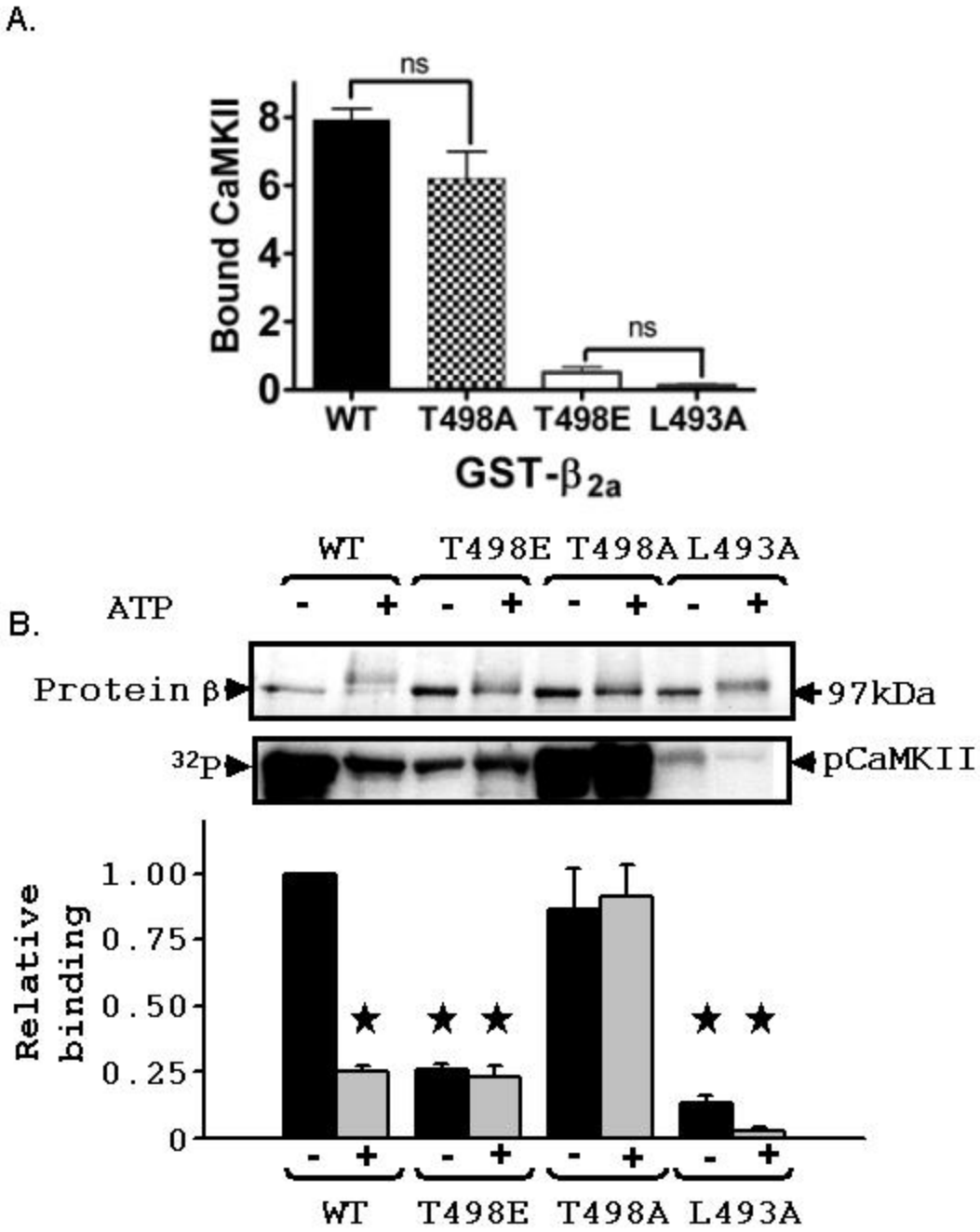


Figure 19. Phosphorylation of β_{2a} Thr498 negatively regulates CaMKII interaction.

A. Glutathione plate binding assays using wild type and mutant GST-β_{2a} subunits. B. Preincubation of GST-β_{2a} mutants with CaMKIIδ2 in the presence or absence of ATP. Protein staining is depicted in the top panel. The middle panel is a representative autoradiograph from the ^{32}P CaMKIIδ2 overlay assay. The graph represents the quantitative results from three experiments. (error bars are $\pm$S.E.M.) (Experiments in panel A were performed by Sunday Abiria)

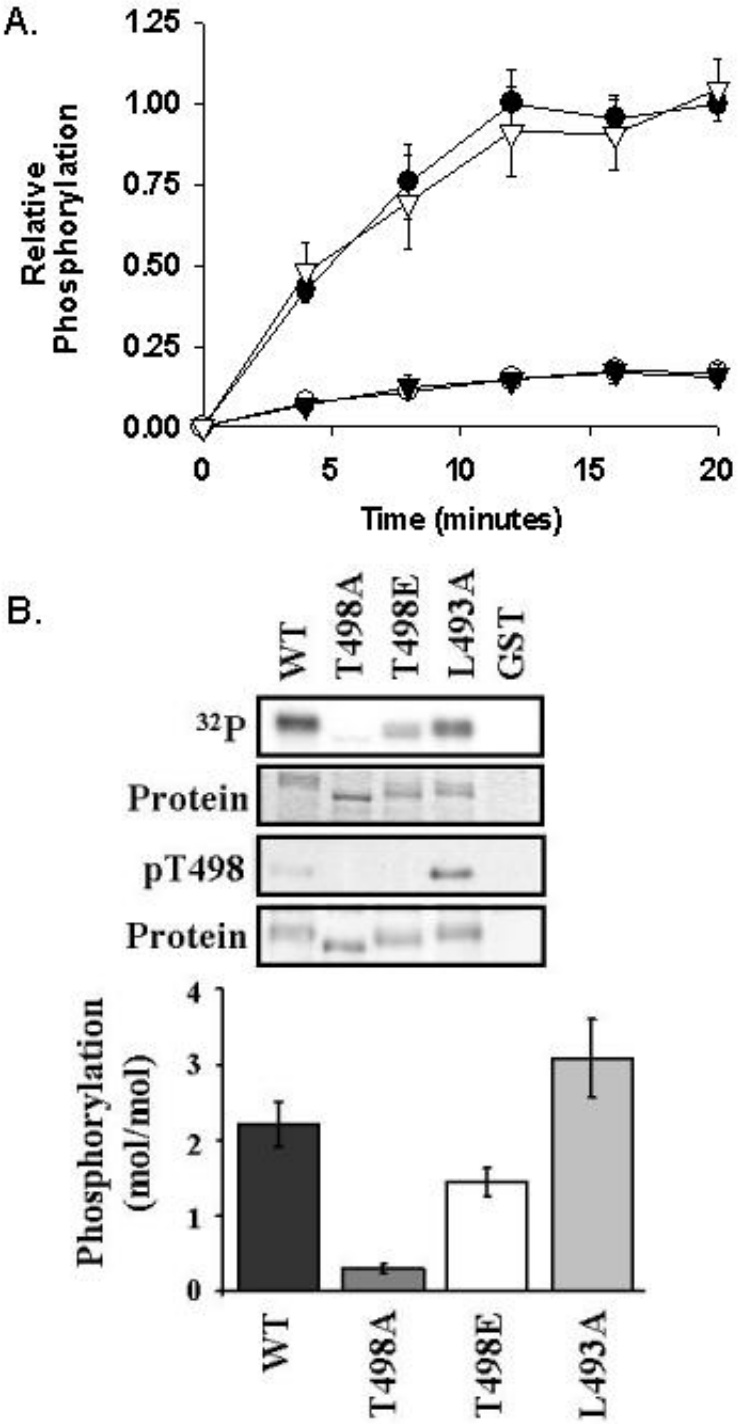


Figure 20. β_{2a} phosphorylation by CaMKII is independent of its interaction with this protein.

A. Initial rate of β_{2a} phosphorylation by CaMKII is strongly dependent on Thr498 (wild type●, Thr498Ala▾, Thr498Glu○, Leu493Ala▿). (error bars are ±S.E.M.) n=3-4. B. CaMKII phosphorylation of β_{2a} is reduced when Thr498 is mutated. The top panel is a representative autoradiograph with the protein stain directly below. The third panel is a representative western blot demonstrating Thr498 phosphorylation in β_{2a}. The protein stain is shown below.

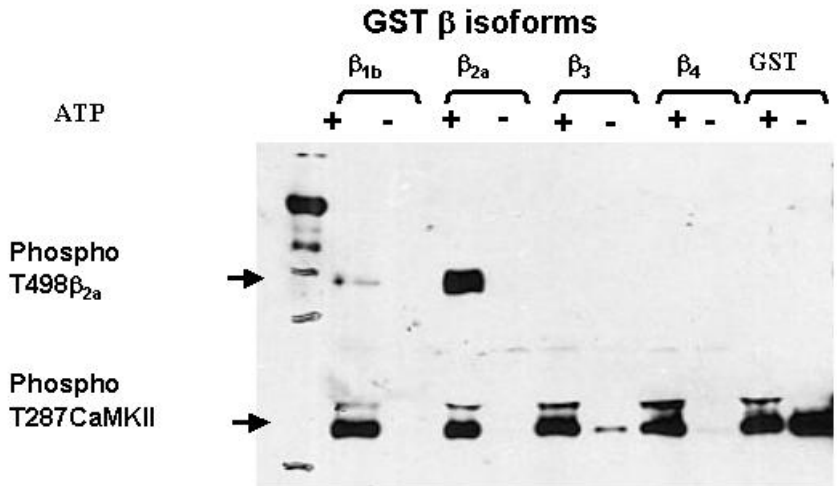


Figure 21. CaMKII phospho Thr287 antibody cross reacts with β_{2a}.
Western blot of *in vitro* phosphorylated GST-β subunits using the Promega active CaMKII antibody at Thr287.

surrounding Thr498 in β_{2a} and Thr287 in CaMKIIδ. A commercially available phospho-Thr287 antibody specifically detected CaMKII phosphorylated β_{2a}, but not non phosphorylated β_{2a}. The phospho-Thr287 antibody did not detect β_3 or β_4 before or after CaMKII phosphorylation, and only weakly detected phosphorylated β_{1b}.

We then used the phospho-Thr498 antibody to probe CaMKII phosphorylation of wild type and mutated β_{2a} proteins. The phosphorylated wild type protein was effectively detected, but the Thr498Ala or Thr498Glu proteins were not detected, demonstrating that the antibody specifically recognized phospho-Thr498. Moreover, the phosphorylated Leu493Ala β_{2a} protein was detected by the phospho-Thr287 antibody at least as efficiently as the phosphorylated wild type β_{2a} protein (Figure 20b). These data demonstrate that Leu493Ala mutation neither affects the initial phosphorylation of β_{2a} at Thr498 nor does it affect total phosphorylation *in vitro*.

CaMKII interaction with β_{2a} is regulated in HEK cells

We have demonstrated the interaction between CaMKII and β_{2a} is regulated by phosphorylation *in vitro*. We next co-expressed CaMKII with FLAG-tagged β_{2a} Thr498Ala, Thr498Glu proteins in HEK293 cells to test whether CaMKII association is regulated by phosphorylation *in situ*. In addition, we expressed the Leu493Ala mutant to determine the role of this residue on the interaction in intact cells. Immunoprecipitations using FLAG antibodies resulted in the co-precipitation of CaMKII from cell lysates containing FLAG

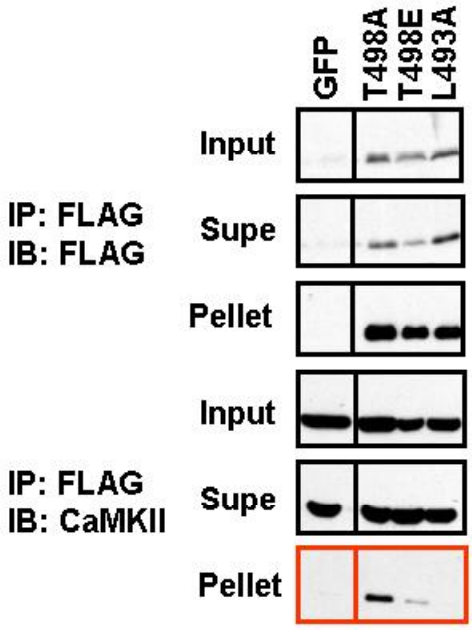


Figure 22. CaMKII interaction with β_{2a} is regulated by Thr498 phosphorylation *in situ*.
The left panel displays the input, supernatant and pellets from immunoprecipitations of FLAG-β_{2a} Thr498Ala, Thr498Glu and Leu493Ala. The right panel shows results from the same membrane probed with a CaMKII antibody. Data are representative of 4 experiments

-β_{2a}(Thr498Ala) and a reduced interaction with Thr498Glu, but not from lysates containing FLAG-β_{2a}(Leu493Ala) (Figure 22). Co-immunoprecipitation of CaMKII with FLAG-β_{2a}(WT) was variable, which is consistent with a regulated interaction (data not shown). These findings further demonstrate a regulated interaction between CaMKII and the β_{2a} subunit *in situ*.

Discussion

VGCCs regulate Ca^{2+} entry in a diverse range of cell types including both excitable and non-excitable cells. New studies revealing the regulation of VGCCs by posttranslational modification and by protein-protein interactions are continuing to be reported. In the present study, we demonstrate CaMKII interactions with β subunits of VGCC containing the LXRXXS/T motif, specifically β_{1b} and β_{2a} but not β_3 or β_4. Further analysis demonstrates that the interaction between β_{2a} and potentially β_{1b} is regulated by CaMKII phosphorylation of Thr498 both *in vitro* and *in situ*.

The modulation of VGCC by the auxiliary β subunits is still being defined. Recent reports demonstrate that the primary docking site for β on the α_1 subunit I-II linker is necessary for membrane expression of N-type Ca^{2+} channels. However, the β subunit modulation of the biophysical properties of the VGCCs appears to occur via secondary interactions (Butcher et al., 2006; Leroy et al., 2005). A third and potentially equally important role for the β subunits as scaffolding proteins has emerged (Beguin et al., 2006; Finlin et al., 2006; Hohaus

et al., 2002). Signaling proteins such as REM GTPases bind to and inhibit VGCCs via the β subunit (Beguin et al., 2006; Finlin et al., 2006). Other proteins including AHNAKs function as large structural proteins linking the VGCC complex to the actin cytoskeleton via the β subunit (Hohaus et al., 2002).

Our previous work demonstrated that CaMKII colocalized with cardiac LTCCs and that β_{2a} Thr498 is necessary for CaMKII-dependent facilitation of Ca^{2+} current (Grueter et al., 2006). The implications of this work are several fold. First, CaMKII localization and potentially regulation of VGCC could be dependent on the β subunit isoform bound to the channel complex. Second, CaMKII activation and phosphorylation of β_{2a} or β_{1b} leads to a decrease in CaMKII association. However, our results suggest that CaMKII interaction with β_{2a} does not have an effect on the initial rate of Thr498 phosphorylation *in vitro,* which raises the question of the role of CaMKII interaction with the β_{2a} subunit. Transduction of the β_{2a} Thr498Ala mutant ablates CaMKII-dependent facilitation of I_{Ca} in cultured adult cardiac myocytes (Grueter et al., 2006). Whether or not phosphorylation of the Thr498 site is directly responsible for the change in the biophysical properties of the LTCCs or Thr498Ala binds CaMKII in a constitutive manner not enabling it to dissociate remains unanswered. Dissociation of activated CaMKII may allow it to phosphorylate nearby substrates such as the recently reported sites on the α_{1c} subunit (Erxleben et al., 2006; Hudmon et al., 2005; Lee et al., 2006). All of these publications co-transfected the β_{2a} subunit;

thus, the effects via α_1 may be β dependent or β specific. Dissociation of CaMKII would also allow protein phosphatases access to the Thr498 site, thus enabling it to dephosphorylate Thr498. Further analysis of the dynamics of this interaction are needed to form a more complete model of how these proteins might play a role in channel regulation.

VGCCs are portals for Ca^{2+} entry and provide a signaling mechanism for translating changes in membrane potential into biochemical responses. The signaling molecules associated with the VGCC complex would therefore be important in modulating the downstream signaling effects. For example, VGCC dependent long term potentiation in dendritic spines is thought to involve cross talk between L-type Ca^{2+} channels and R-type Ca^{2+} channels mediated by activation of CaMKII (Yasuda et al., 2003). Both types of VGCC are regulated by β subunits and depending on the β subunit isoform associated with the complex CaMKII mediated signaling may vary. Precise regulation of Ca^{2+} signaling proteins in distinct microdomains may provide a mechanism for signal specificity.

Alterations in β subunit composition occur in disease, therefore modifying signaling/regulation of VGCC. Bodi et. al. proposed that β subunit channelopathies may be a source of heart failure (Bodi et al., 2003). The work shown here may represent one of the physiological mechanisms cells use to adapt to changes in their local environment.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Summary

The original goal of my thesis project was to identify and characterize the potential role of the VGCC β_{2a} subunit in mediating CaMKII-dependent facilitation of I_{Ca} in cardiac myocytes. The proposed plan was to identify CaMKII phosphorylation sites within the β_{2a} subunit using biochemical and proteomic approaches. Electrophysiological techniques would be used to screen mutants for functionally significant sites using a heterologous cell system. Following the successful identification of functionally significant CaMKII phosphorylation sites within β_{2a} in model cells, we would use viral mediated gene delivery in cultured adult cardiac myocytes to test the functional consequence of mutating the identified site(s) within β_{2a} on CaMKII dependent I_{Ca} facilitation.

In theory the proposed experiments and interpretations were logical; however, many unknowns and technical difficulties were evident. First and foremost, evidence for β subunit involvement in CaMKII-dependent facilitation was preliminary. Second, there were 99 Ser/Thr residues out of 604 total amino acids in β_{2a}, each representing a potential CaMKII phosphorylation site. Many of

the technical requirements necessary for the success of this project were not in place at the outset. Therefore, much time was spent developing new technologies and refining old techniques that would directly facilitate progress. Among these was the isolation and culture of adult cardiac myocytes. Upon successful culture of the terminally differentiated cardiac cells another challenge arose. Introducing an exogenous protein in terminally differentiated cells. A lentiviral approach carrying GFP-IRES-β_{2a} was used to transduce cardiac myoctyes. Previous studies by Wei et al and Colecraft et al showed that the β subunit is the rate limiting step in LTCC functional expression at the surface of the plasma membrane and importantly, that exogenous β subunits act in a dominant manner, a fortunate biological phenomenon that made completing my project possible (Colecraft et al., 2002; Wei et al., 2000).

Ultimately, β_{2a} Thr498 was identified as a significant CaMKII phosphorylation site by proteomic and biochemical methods. Mutating Thr498 to Ala abrogated CaMKII-dependent increases in single channel open probability in transiently transfected heterologous cells. Mutating this site in β_{2a} ablated CaMKII dependent facilitation in cultured adult cardiac myocytes. Immunohistochemical analysis revealed β_{2a} and CaMKII colocalization at Z-lines in cardiac myocytes. I also recognized that the sequence surrounding Thr498 is similar to the CaMKII autoregulatory domain and the CaMKII binding site in the NR2B subunit of the NMDA receptor. This led to studies showing that β_{2a} and β_{1b} are CaMKAPs. Further analysis of the interaction demonstrated that binding is negatively regulated by Thr498 phosphorylation.

In summary, I have identified a mechanism for CaMKII dependent facilitation of I_{Ca} in cardiac myocytes that is mediated by the regulatory β_{2a} subunit as proposed. In addition I have characterized a mechanism for CaMKII localization to LTCC in cardiac myocytes and the intricate modes of cross talk between β_{2a} and CaMKII. Taken together this work provides a strong foundation for future work involving CaMKII regulation of Ca^{2+} signaling in diverse cell types.

Future directions

CaMKII-dependent I_{Ca} facilitation

Our data strongly suggest that CaMKII regulates LTCCs via β_{2a} Thr498. While functional studies using the dominant negative β_{2a} Thr498Ala mutant in adult cardiac myocytes to ablate I_{Ca} facilitation strongly support this statement, the actual mechanism for this regulation remains elusive. There are at least two potential modes of regulation. One mode is that β_{2a} Thr498 phosphorylation directly regulates the biophysical properties of the channel. A second possibility is that β_{2a} Thr498 phosphorylation enhances CaMKII dissociation, therefore releasing CaMKII to phosphorylate an additional site(s) such as the recently identified sites on the C-terminus of the α_{1c} subunit (Figures 23 and 24). Experiments have been designed to address the question of the mode of regulation using the β_{2a} Leu493Ala mutant. Biochemical analysis of the Leu493Ala mutant in Chapter IV shows that Thr498 phosphorylation is not altered, but that CaMKII interaction is significantly reduced. Assuming it would

function in a dominant fashion when transduced into adult cardiac myocytes, as did the Thr498Ala mutant, ablation of I_{Ca} facilitation would support our first hypothesis. However, if I_{Ca} facilitation was the same as with wild type β_{2a} it would support our second hypothesis. One caveat is that the Leu493Ala mutant might reduce CaMKII localization to the LTCC complex, thus reducing CaMKII regulation of the channel. In any event, the experiment would provide evidence for the importance of the β_{2a} subunit in mediation of CaMKII localization to the LTCC complex.

Deciphering the physiological role of CaMKII-dependent facilitation

It has been postulated that the physiological role for CaMKII-dependent I_{Ca} facilitation is to allow for an increase in the force of contraction upon increase in the frequency of stimulation (Bers and Guo, 2005; Pitt et al., 2006). However, direct evidence was lacking because the mechanism for CaMKII-dependent facilitation was unknown. Based on the results presented in this thesis, a transgenic mouse model with β_{2a} Thr498 mutated to Ala may provide a tool to test this hypothesis.

Another potential experiment to address this hypothesis is to evaluate whether or not β_{2a} Thr498Ala alters cardiac myocyte contractility under various conditions. The established culture conditions and transductions could be

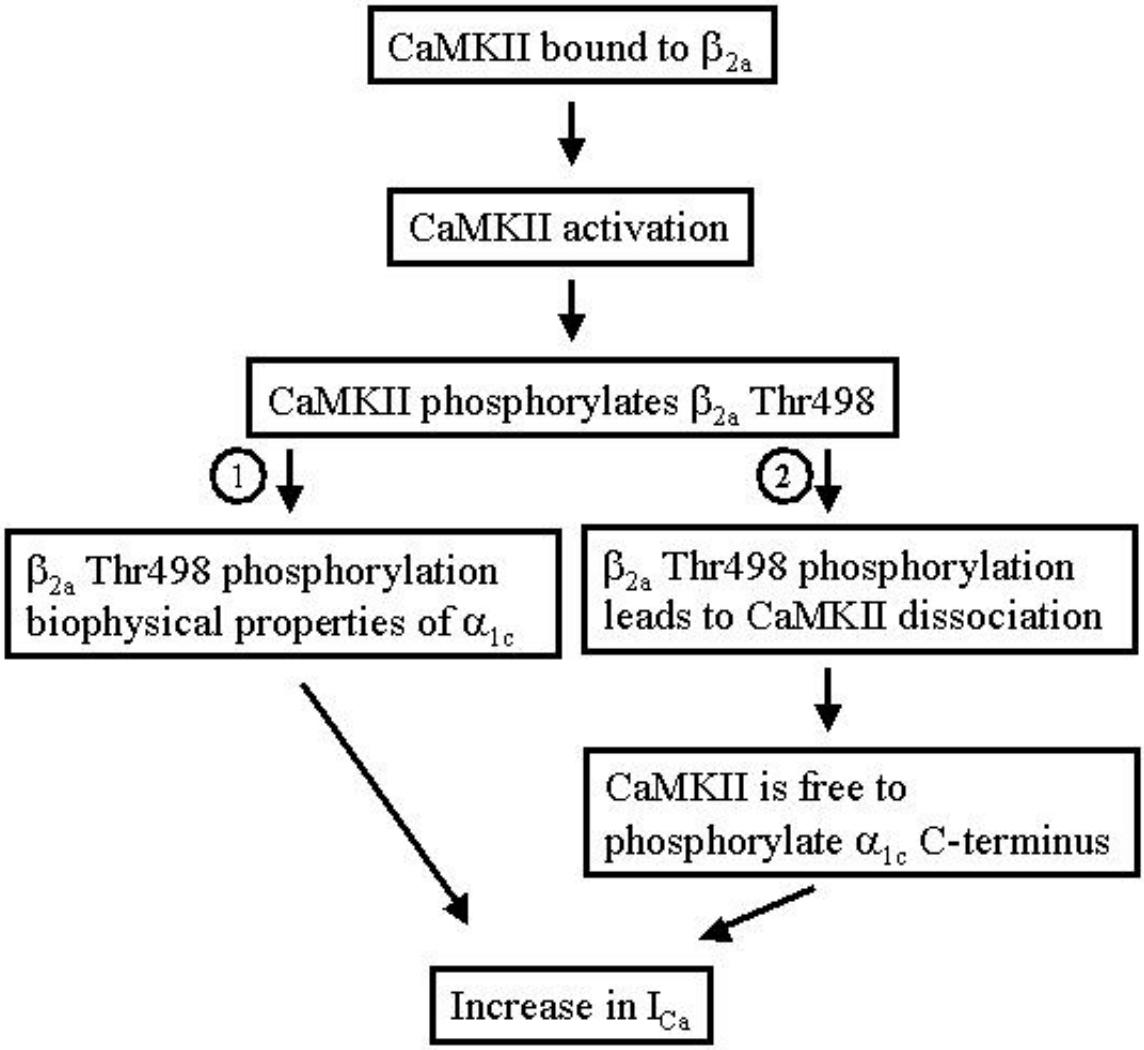


Figure 23. Flow chart representing multiple interpretations for the β_{2a} dependent CaMKII facilitation of LTCC I_{Ca}.
CaMKII associated with the LTCC complex becomes activated and phosphorylates β_{2a} Thr498. Ablation of this site abolishes CaMKII dependent facilitation that could potentially occur by two different mechanisms (arm 1 vs. arm 2 in the diagram). First, Thr498 phosphorylation could directly regulate the biophysical properties of the channel enhancing I_{Ca} directly. Another potential mechanism is Thr498 phosphorylation leads to CaMKII dissociation freeing CaMKII to phosphorylate an additional target such as the α_{1c} C-terminus, ultimately resulting in LTCC I_{Ca} facilitation.

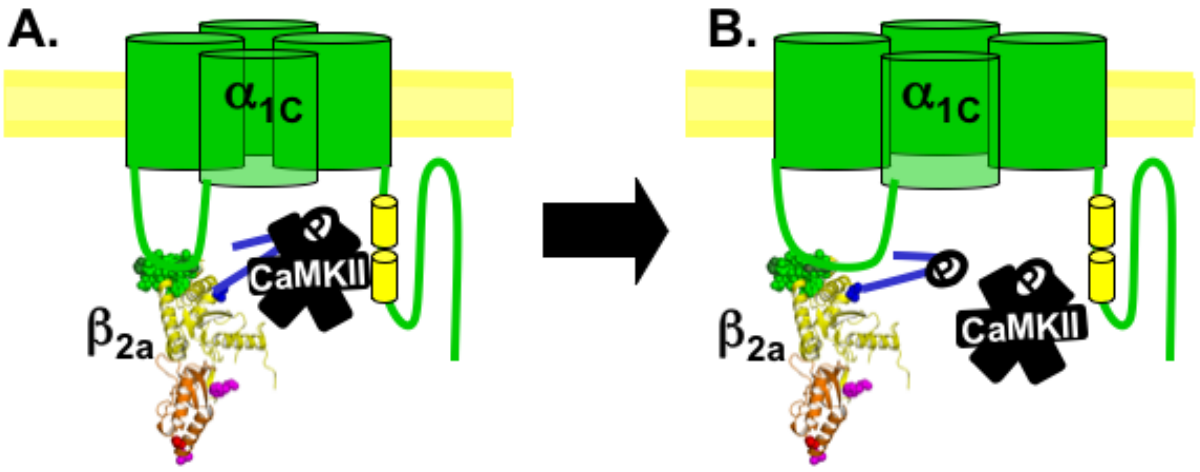


Figure 24. Schematic diagram representing CaMKII dependent facilitation of LTCC.

A. Activated CaMKII can associate with the C-terminus of the LTCC β_{2a} subunit and with the C-terminus of the α_{1C} subunit. B. Phosphorylation of β_{2a} at Thr498 is sufficient for CaMKII dependent facilitation of I_{Ca} in cardiac myocytes.

utilized. Experimental parameters could include altering the pacing frequency, β-adrenergic stimulation and pharmacological inhibition of CaMKII. We would anticipate an increase in peak contraction following CaMKII activation. A decrease in peak contraction compared to wild type would suggest that the β_{2a} Thr498 plays a functional role in regulating the force-frequency relationship.

Both CaMKII and the LTCC have been implicated in generating EADs during the cardiac action potential plateau phase. Early-afterdepolarizations are triggers for cardiac arrhythmias (Anderson, 2005). To determine if CaMKII mediated EADs are a result of β_{2a} Thr498 phosphorylation, experiments transducing β_{2a} Thr498Ala could be performed and analyzed by current clamp. We would predict that, if this site is a trigger point for EADs, eliminating the site would significantly reduce EADs when compared to wild type.

Other CaMKII phosphorylation sites on β_{2a}

The major goal of this work was to identify a mechanism for CaMKII-dependent facilitation involving the β_{2a} subunit. Other potential regulatory processes may be occurring via CaMKII phosphorylation that was not directly addressed. The *in vitro* data show that Thr498 is the initial phosphorylation site but that other sites do exist. A short list of sites identified by mass spectrometry are included in Figure 11. These sites include a PKA phosphorylation site, Ser459, that has no known function (Bunemann et al., 1999). Another potential CaMKII site is Ser574. Phosphorylation of this site by PI3 kinase enhances membrane expression of the VGCC complex (Viard et al., 2004). Other

unidentified phosphorylation sites may play an important role in regulating the association of other regulatory proteins with the β_{2a} subunit.

LTCC complex I_{Ca} "remodeling" in disease states

The predominant β subunit expressed in the heart is thought to be the β_2 isoform. However, evidence suggests that β subunit expression may vary depending on the disease state of the heart with an increase in β_3 expression with the LTCC complex. Many questions arise when considering the differences between β_{2a} and β_3 in the experiments done in Chapter IV. First, does the β_3 subunit (or β_{1b} and β_4) support facilitation in the same manner as β_{2a}? The sequences are quite similar at the CaMKII phosphorylation motif; however, one significant difference is a Pro at the -5 position where β_{2a} has a Leu. The β_3 subunit does not bind CaMKII when compared to β_{2a}; however, it is an excellent substrate. Overexpressing β_3 in cultured adult myocytes in the same manner as done with β_{2a} may provide evidence for the mechanism of CaMKII-dependent facilitation. More experiments to support that CaMKII phosphorylates the homologous motif and the differences in the intrinsic regulatory properties of the LTCC are needed.

CaMKII regulation of HVA Ca^{2+} channels

The work described here has focused on CaMKII-dependent regulation of LTCCs via the β_{2a} subunit in model cells and in adult cardiac myocytes. The LTCC complex is expressed in many cell types including neurons, pancreatic

beta cells and chromaffin cells. Its functional role is continuing to be evaluated as well as its regulation by CaMKII. We hypothesize that the mechanism for CaMKII regulation of LTCC in myocytes occurs in other cell types as well. The major caveat is that the electrophysiological readout for CaMKII regulation of LTCC in myocytes is dependent on SR Ca^{2+} release. An experimental protocol specific for CaMKII regulation in each cell type may prove to be technically challenging. In addition to regulating LTCCs there is increasing evidence for CaMKII regulation of other HVA Ca^{2+} channels (Yasuda et al., 2003). The same caveat exists for the other HVA Ca^{2+} channels as does for the LTCCs in other cell types. T-type Ca^{2+} channels (low voltage-activated channels) have been shown to bind to and be modulated by CaMKII directly (Barrett et al., 2000; Welsby et al., 2003; Wolfe et al., 2002). CaMKII binds in the II-III linker of the α_1 subunit in a phosphorylation dependent manner and regulates the channel by phosphorylation of the loop directly (Yao et al., 2006). In T-type Ca^{2+} channels, this intracellular loop is much longer and is thought to compensate for the role the β subunit plays in regulation of HVA Ca^{2+} channels. Ultimately, the role of CaMKII regulation of VGCC in normal and disease states may provide useful therapeutic targets for treatment.

CaMKII binding/phosphorylation motif and its implications

The novel regulatory site identified on β_{2a} is highly homologous to the CaMKII autoregulatory domain and the NR2B subunit of the NMDA receptor. All three are excellent substrates and bind directly to CaMKII. The existence of

three homologous domains suggests the possibility that other proteins may have similar domains and may have an important role in Ca^{2+} signaling. Using this motif it may be possible to identify and characterize other CaMKAPs in different signaling pathways. Table 1 contains the results of a blast search with select proteins containing a sequence homologous to the CaMKII binding domain. The exact amino acids involved in the interaction are unknown. A more defined binding motif would allow for a directed approach to identifying potential novel CaMKAPs. Some work using traditional site directed mutagenesis of residues thought to be involved in the interaction between CaMKII and the β_{2a} subunit and the NR2B subunit has been done in our lab (Strack et al., 2000a). Further characterization of the domain could be achieved by peptide mapping using synthetic peptides with specific amino acid substitutions. Ultimately, identification of CaMKAPs utilizing the binding motif outlined here may parallel that of AKAPs, thus providing spatio-temporal regulation of Ca^{2+} signaling mediated by CaMKII.

CaMKAP's regulate CaMKII activation

Up to this point we have shown CaMKII co-localization with and regulation of LTCCs are mediated via the β_{2a} subunit. A major question remains as to whether or not the β_{2a} subunit can reciprocate by regulating CaMKII activity. Previous studies in our lab and others have shown that the NR2B interaction with CaMKII can enhance or inhibit CaMKII activity *in vitro* (Bayer et al., 2001; Robison et al., 2005). Preliminary studies with β_{2a} suggest that it can also regulate CaMKII activity in a similar manner as seen with NR2B. Preliminary

Table 1. Blast search for CaMKII phosphorylation/ binding motif

	Name of protein	sequence
	Phosphorylation motif/binding motif	
1	spectrin alpha chain	TLLTKQETFD
2	mitochondrial ribosomal protein S5	RGLSRQETHQ
3	zinc finger protein 592	KNTSRQESFE
4	Sac domain-containing inositol phosphatase 3 variant	MTQNRQESFD
5	plasminogen activator	FSLPRQETYR
6	SNF1-like kinase 2	LPLPRQETPP
7	SH3 and multiple ankyrin repeat domains 2 isoform 1	GPLRRQETEN
8	TATA element modulatory factor 1	SASSRQETTD
9	NF-kappa B inhibitor	HFPAFQETVD
10	Src-like adapter protein SLAP	PVTLRQKTFD
11	neuron navigator 2 isoform 2	LRIRRQHSSD
12	similar to von Willebrand factor	PDLHRQHSD
13	regulating synaptic membrane exocytosis 1	DRMHRQRSPT
14	rab3 interacting protein variant 2	ERMHRQRSPT
15	dystrophin, muscular dystrophy	PQKKRQITVD
		DLRQRQISVD
	binding motif	
16	MHC class I antigen	LRGYRQHAYD
17	huntingtin interacting protein 1-related	LGELRRQHVL
18	nuclear factor kappa-B, subunit 1	KELIRQAALQ
19	laminin, beta 4	LNLSRQAKAD
20	cardiotrophin-like cytokine	RGLNRQAATA
21	cyclin I variant	TDLSRQEGHA
22	four domain-type voltage-gated ion channel alpha-1 subunit	LGLSRQELGY
23	V-crk sarcoma virus CT10 oncogene homolog	GRLSRQEAVA
24	myelin expression factor-3	AELSRQEAPK
25	HUMAN Myosin-18B	GFLSRQEFKK
26	epidermal growth factor-receptor-binding protein GRB-3	GRLSRQEAVA
27	voltage gated channel like 1	LGLSRQELGY
28	proteinase inhibitor, clade I (neuroserpin)	LVLSRQEVPL
29	apoptosis inducing factor	CSLIRQNGNF
30	HDAC 4	ELLFRQQALL

data show that β_{2a} significantly inhibits CaMKII activity (data not shown). Recently we have uncovered evidence that the presence of a Thr498 phosphorylation-competent GST-β_{2a} subunit is able to enhances CaMKII autophosphorylation while the phosphorylation-incompetent mutants inhibit CaMKII autophosphorylation (Figure 25). CaMKII can autophosphorylate at many sites under the assay conditions used; however, preliminary evidence suggests that Thr287 phosphorylation is not altered (Figure 24). Interestingly, using antibodies generated to phospho-Thr305/6, autophosphorylation appears to be altered in a manner consistent with the results from the quantification of the autoradiographs (Figure 25). CaM binding to CaMKII is inversely regulated by Thr305/6 phosphorylation, termed CaM "capping". Additionally, Thr287 autophosphorylation enhances CaM association with CaMKII by 1000 fold in a process termed CaM trapping (De Koninck and Schulman, 1998; Hudmon and Schulman, 2002). One interpretation of the enhanced Thr305/6 autophosphorylation in the presence of the phosphorylation competent β_{2a} is that association of the CaMKAP in some way reduces CaM association.

Upon activation, CaMKII binds β_{2a}. In this state CaMKII is inhibited. Upon phosphorylation of Thr498, the complex dissociates. However, the mechanism could be due directly to phosphorylation at Thr498 or due to the dissociation of CaMKII, thus reducing CaM affinity and increasing the probability of phosphorylation at Thr305/6. Additional phosphorylation sites with unrecognized roles could also be involved in regulating CaM affinity and CaMKII autophosphorylation as well (Figure 26). Further experiments testing the

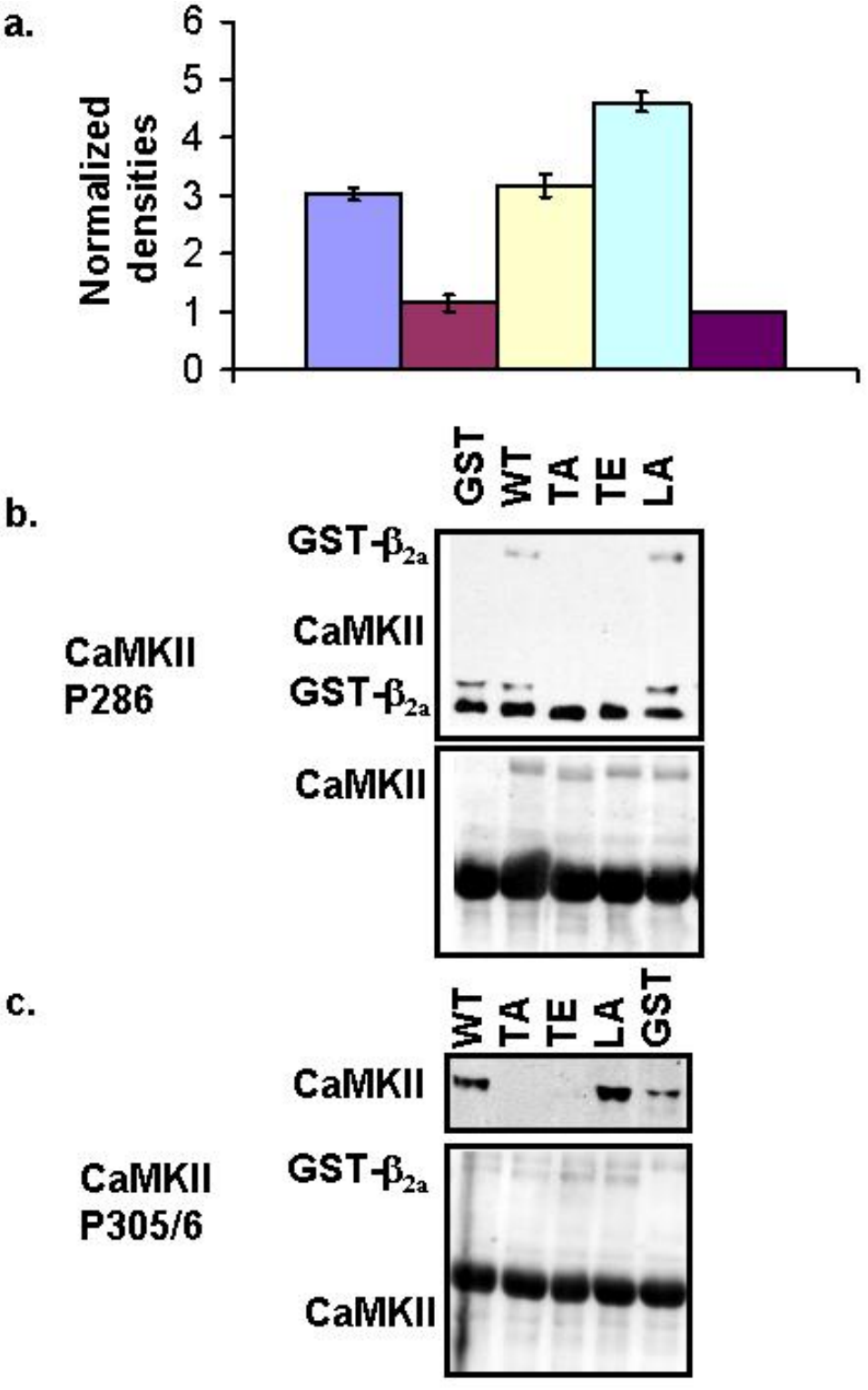

a.
Normalized densities
0
1
2
3
4
5
6
b.
GST
WT
TA
TE
LA
GST-β2a
CaMKII
GST-β2a
CaMKII
P286
CaMKII
c.
WT
TA
TE
LA
GST
CaMKII
GST-β2a
CaMKII
P305/6
CaMKII

Figure 25. CaMKII autophosphorylation is enhanced in the presence of GSTβ_{2a} mutants.
A. Incubation of GST-β_{2a} wild type (dark blue), Thr498Ala (red), Thr498Glu (yellow) or Leu493Ala (light blue) with CaMKII regulates CaMKII autophosphorylation. ^{32}P incorporation into CaMKII at 40nM in the presence of 1000nM GST protein was quantified by pixel density using a phospho-imager. Experiments were normalized to GST (purple). (n=4, error bars are ±S.E.M.) B. Western blot of *in vitro* phosphorylation assay containing wild type and mutant β subunits using phospho Thr286 CaMKII antibody. The CaMKII phospho Thr286 antibody cross reacts with phosphorylated β_{2a} Thr498. C. Western blot from the same assay as in panel b using the CaMKII phospho Thr305/6 antibody.

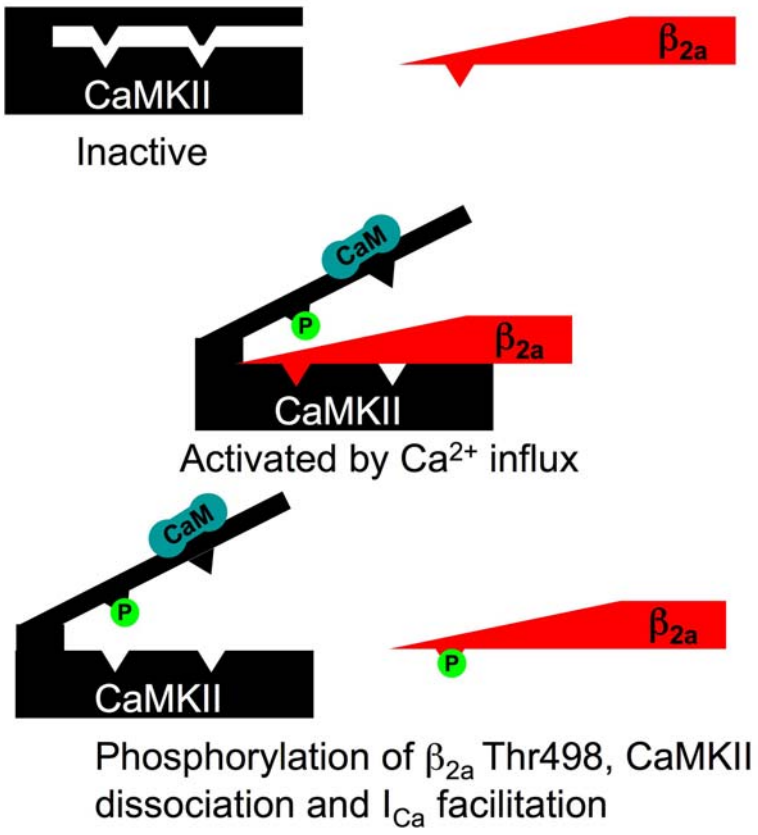


Figure 26. Proposed model of CaMKII interaction with and regulation by β_{2a}. Activated CaMKII interacts with β_{2a} thus inhibiting CaMKII activity. Phosphorylation of β_{2a} at Thr498 releases β_{2a}.

proposed change in CaM affinity in the presence of wild type β_{2a} compared to Thr498Ala β_{2a} may provide insight into the biochemical mechanism observed. The physiological role for feedback regulation of the CaMKAP on CaMKII itself is uncertain, but we speculate that it is a mechanism for keeping CaMKII activation and regulation of Ca^{2+} entry either via LTCC or the NMDA receptor in check.

Final Summary

The results of this thesis suggest that the β subunit is a key mediator of CaMKII dependent regulation of cardiac LTCCs. We have defined a novel mechanism for CaMKII interaction with and regulation of the LTCC via phosphorylation of β_{2a} Thr498. The binding/regulatory site is conserved in β_{1b} but not in β_3 nor β_4. Together this provides a more comprehensive model for CaMKII dependent regulation of LTCCs and potentially other HVA Ca^{2+} channels, CaMKII interaction with its targets and Ca^{2+} cycling within a cardiac myocyte. Future work based on these findings may identify a potential pharmacological target for the treatment of heart disease.

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