Cardiomyocyte Calcium and Calcium/Calmodulin-dependent Protein Kinase II: Friends or Foes?

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ABSTRACT

Calcium (Ca^{2+}) is a critical second messenger in cell signaling. Elevated intracellular Ca^{2+} can activate numerous Ca^{2+} -regulated enzymes. These enzymes have different subcellular localizations and may respond to distinct modes of Ca^{2+} mobilization. In cardiac muscle, Ca^{2+} plays a central role in regulating contractility, gene expression, hypertrophy, and apoptosis. Many cellular responses to Ca^{2+} signals are mediated by Ca^{2+} /calmodulin-dependent enzymes, among which is the Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII). Putative substrates for CaMKII include proteins involved in regulating Ca^{2+} storage and release, transcription factors, and ion channels. The major isoform of CaMKII in the heart is CaMKII δ . Two cardiac splice variants, CaMKII δ_B and δ_C , differ in whether they contain a nuclear localization sequence. Our laboratory has examined the hypothesis that the nuclear δ_B and the cytoplasmic δ_C isoforms respond to different Ca^{2+} stimuli and have distinct effects on hypertrophic cardiac growth and Ca^{2+} handling. We have shown that pressure overload-induced hypertrophy differentially affects the nuclear δ_B and the cytoplasmic δ_C isoforms of CaMKII. Additionally, using isolated myocytes and transgenic mouse models, we demonstrated that the nuclear CaMKII δ_B isoform plays a key role in cardiac gene expression associated with cardiac hypertrophy. The cytoplasmic CaMKII δ_C isoform phosphorylates substrates involved in Ca^{2+} handling. Dysregulation of intracellular Ca^{2+} and resulting changes in excitation-contraction coupling characterize heart failure and can be induced by *in vivo* overexpression of CaMKII δ_C and phosphorylation of its substrates. The differential location of CaMKII isoforms and their relative activation by physiological vs. pathological stimuli may provide a paradigm for exploring and elucidating how Ca^{2+} /CaMKII pathways can serve as both friends and foes in the heart.

I. Intracellular Ca^{2+} Regulation

As a second messenger, Ca^{2+} regulates acute physiological functions, including contraction of cardiac, skeletal, and smooth muscle and release of hormones and neurotransmitters. Ca^{2+} also regulates more-chronic cellular responses, including cell proliferation and cell survival. Dysregulation of intracellular Ca^{2+} homeostasis can lead not only to loss of normal physiological control mechanisms but also to pathological changes in cell growth.

Intracellular cytosolic Ca^{2+} concentrations are regulated carefully to remain at ≈ 100 nM under resting conditions. This occurs even in the face of mM levels of Ca^{2+} in the extracellular space and high Ca^{2+} in intracellular organelles such

as the endoplasmic reticulum (ER). Acute increases in Ca^{2+} are needed to elicit physiological responses and are achieved through the actions of hormones or neurotransmitters on cell-surface receptors. In smooth muscle and secretory glands, ligands such as norepinephrine, acetylcholine, endothelin-1, and angiotensin II stimulate G protein-coupled receptors (GPCR) coupled to Gq to activate phospholipase C (PLC), catalyze phosphatidylinositol biphosphate (PIP_2) breakdown, and generate inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes Ca^{2+} from the ER/sarcoplasmic reticulum (SR), transiently increasing Ca^{2+} . In skeletal muscle, nicotinic receptor activation by acetylcholine depolarizes the membrane potential to induce Ca^{2+} release from the SR. In cardiac muscle, Ca^{2+} transients occur with every heart beat. The amplitude of these transients is increased via stimulation by norepinephrine of β -adrenergic receptors coupled to Gs, which activate adenylate cyclase to increase cyclic adenosine monophosphate (cAMP). Subsequent activation of protein kinase A (PKA) phosphorylates Ca^{2+} regulatory proteins such as voltage-dependent Ca^{2+} channels and phospholamban (PLB), leading to increased Ca^{2+} influx and releasable SR Ca^{2+} .

II. Ca^{2+} -regulated Enzymes and Ca^{2+} /Calmodulin-dependent Protein Kinase

Once neurotransmitters or hormones elevate Ca^{2+} , any of a number of Ca^{2+} -regulated enzymes — including protein kinases, protein phosphatases, phospholipases, nitric oxide synthases, cysteine protease calpains, and endonucleases — can be activated. For some of these, Ca^{2+} induces activation by binding to calmodulin (CaM), an intracellular Ca^{2+} sensor. Three Ca^{2+} /calmodulin-dependent enzymes have significant roles in cardiac function: Ca^{2+} /calmodulin-dependent protein kinase (CaM kinase or CaMK), protein phosphatase 2B (calcineurin), and myosin light chain kinase (MLCK). In contrast to CaMK and calcineurin, which have broad substrate specificities, MLCK is a dedicated enzyme that phosphorylates only the regulatory light chain of myosin II, modulating Ca^{2+} sensitivity of myofilaments and cardiac contractility (Sweeney *et al.*, 1993) and sarcomere organization during cardiac hypertrophy (Aoki *et al.*, 2000). This review will consider the role of Ca^{2+} -dependent enzymes in regulating gene expression, Ca^{2+} handling, and apoptosis, focusing primarily on CaMK.

CaMKs are ubiquitous mediators of Ca^{2+} signaling (Braun and Schulman, 1995). This multifunctional serine/threonine family, consisting of CaMKI, -II, and -IV, has an extremely wide tissue distribution and is represented to varying degrees in all eukaryotic systems examined. Studies carried out over the past decade demonstrate that CaMKs can phosphorylate multiple substrates and regulate numerous cellular functions. CaMKI and CaMKIV are monomeric

enzymes that are activated by phosphorylation through an upstream kinase (CaMK kinase) (Lee and Edelman, 1994; Tokumitsu *et al.*, 1995). These isoforms are expressed at very low levels in the heart (Edman and Schulman, 1994; Colomer *et al.*, 2003). In contrast, CaMKII, the major cardiac isoform, is a multimer of 6–12 subunits encoded by four separate genes: α , β , γ , and δ (Braun and Schulman, 1995). Binding of Ca^{2+} /CaM to CaMKII leads to its activation and subsequent autophosphorylation, rendering it autonomous (i.e., active) in the absence of Ca^{2+} /CaM (Braun and Schulman, 1995). Several laboratories have demonstrated that the δ subunit of CaMKII predominates in the heart and that distinct splice variants of CaMKII δ , characterized by the presence of a second variable domain, exist (Edman and Schulman, 1994; Baltas *et al.*, 1995; Mayer *et al.*, 1995). Of particular interest, the δ_B subunit contains an 11-amino acid nuclear localization signal (NLS) that localizes it to the nucleus. In contrast, the δ_C isoform lacks the NLS and localizes to the cytoplasm (Edman and Schulman, 1994; Srinivasan *et al.*, 1994; Ramirez *et al.*, 1997). Heteromultimers comprised predominantly of δ_B subunits (CaMKII δ_B) localize to the nucleus, while those with δ_C (CaMKII δ_C) localize to the cytoplasm (Srinivasan *et al.*, 1994).

III. Role of Ca^{2+} Signaling in Gene Expression and Cardiac Hypertrophic Growth

Ca^{2+} is a well-established regulator of transcriptional changes in gene expression (Hardingham and Bading, 1998). Changes in intracellular Ca^{2+} also have been suggested to mediate cardiac hypertrophic responses (for a review, see Frey *et al.*, 2000). Modulations in Ca^{2+} levels would need to be transmitted to the nucleus to affect transcriptional regulation of genes associated with cardiac hypertrophy. The nuclear isoform of CaMKII (CaMKII δ_B) would, therefore, be the isoform predicted to play a predominant role in Ca^{2+} -mediated transcriptional gene regulation. Both Ca^{2+} and CaM can translocate into the nucleus and could activate nuclear localized CaMKII (Heist and Schulman, 1998). There also is evidence for independent regulation of nuclear Ca^{2+} via IP_3 receptors localized in the cell nucleus (Malviya and Rogue, 1998). CaMK and calcineurin have been shown to play critical and often synergistic roles in transcriptional regulation in cardiomyocytes (Passier *et al.*, 2000) (Figure 1). There is growing evidence that the amplitude, frequency, source, and subcellular localization (spatial and/or temporal modes) of Ca^{2+} signals are determinants of distinct transcriptional responses (Berridge, 1997; Dolmetsch *et al.*, 1997), allowing regulation of diverse cellular processes in response to the same second messenger (Ca^{2+}). In this regard, it may be important to note that CaMK is activated by high and transient Ca^{2+} spikes and its activity is dependent on Ca^{2+} spike frequency

FIG. 1. Calcium (Ca^{2+}) signaling in cardiac gene expression and hypertrophic growth. Ca^{2+} binds to calmodulin (CaM), which, in turn, activates Ca^{2+} /calmodulin-dependent protein kinase (CaMK) or calcineurin. CaMK can phosphorylate histone deacetylase (HDAC) and dissociate it from myocyte enhancer factor 2 (MEF2) and/or serum response factor (SRF), thereby relieving HDAC inhibition of MEF2 and/or SRF transcriptional activity. Calcineurin can dephosphorylate the nuclear factor of activated T cells (NFAT), which enables it to translocate to the nucleus to induce hypertrophic gene expression. Calcineurin also can activate MEF2 through a post-translational mechanism. Inhibition of calcineurin by cyclosporin A (CsA)/FK506, Cain/AKAP79, or modulatory calcineurin-interacting protein (MCIP)1 blocks calcineurin signaling pathways, while inhibition of CaMK by KN-62 or KN-93 blocks CaMK signaling pathways.

(Hudmon and Schulman, 2002), whereas calcineurin responds to low, sustained Ca^{2+} plateaus (Dolmetsch *et al.*, 1997).

A. CAMK SIGNALING IN GENE EXPRESSION AND CARDIAC HYPERTROPHY

1. The Effect of CaMK on Gene Transcription

CaMK has been suggested to regulate gene expression via activation of several different transcription factors, including activation protein 1 (AP-1) (Ho *et al.*, 1996), CAAT-enhancer binding protein (C/EBP) (Wegner *et al.*, 1992), activating transcription factor (ATF-1) (Sun *et al.*, 1996), serum response factor (SRF) (Misra *et al.*, 1994), cAMP-response element binding protein (CREB)

(Sheng *et al.*, 1991), and myocyte enhancer factor 2 (MEF2) (Passier *et al.*, 2000). CaMKII and CaMKIV have been shown to activate CREB by phosphorylation of Ser133 (Matthews *et al.*, 1994). Surprisingly, CREB phosphorylation does not appear to be altered in transgenic mice expressing CaMKII δ_B (Zhang *et al.*, 2002) or CaMKIV (Passier *et al.*, 2000), both of which localize to the nucleus. Therefore, phosphorylated CREB cannot account for the long-term changes in cardiac function in these mice. Another transcription factor, MEF2, is upregulated during cardiac hypertrophy (Kolodziejczyk *et al.*, 1999; Lu *et al.*, 2000) and has been suggested to act as a common endpoint for hypertrophic signaling pathways in the myocardium (Kolodziejczyk *et al.*, 1999; Lu *et al.*, 2000). Studies using transgenic overexpression of CaMKIV demonstrate that MEF2 is a downstream target for CaMKIV (Passier *et al.*, 2000). The mechanism by which CaMK signaling activates MEF2 *in vivo* remains to be determined but recent studies have suggested that MEF2 interacts with class II histone deacetylases (HDACs) and other repressors that normally limit expression of MEF2-dependent genes (Figure 1). CaMKI and CaMKIV activate MEF2 by dissociating HDACs and other repressors (Lu *et al.*, 2000). Most recently, SRF has been shown to be activated by CaMKIV in a similar manner (i.e., by dissociating HDACs) (Davis *et al.*, 2003). Both MEF2 and SRF activation have been demonstrated to occur in response to activation of the noncardiac CaMKI and IV isoforms. However, the ability of CaMKII to regulate HDAC — and thereby activate MEF2 and SRF — has not been explored and the selectivity of the nuclear vs. cytoplasmic CaMKII δ isoforms is undetermined. Our preliminary studies indicate that the nuclear CaMKII δ_B overcomes HDAC5-mediated repression of MEF2 activity, while the cytoplasmic CaMKII δ_C does not (T. Zhang and J.H. Brown, unpublished data). These findings are consistent with our earlier work implicating CaMKII δ_B (vs. δ_C) in control of gene expression (Ramirez *et al.*, 1997).

2. The Role of CaMK in Hypertrophic Growth

Studies from a variety of *in vivo* preparations — including hypertensive rat hearts, coronary artery-ligated rabbit hearts, and transverse aortic-constricted (TAC) mouse hearts — have demonstrated increased CaMKII expression and activity in hypertrophied myocardium (see Table I for a summary) (Currie and Smith, 1999; Boknik *et al.*, 2001; Hagemann *et al.*, 2001; Zhang *et al.*, 2003). Studies using isolated cardiomyocytes and CaMK inhibitors KN-62 or KN-93 also suggested that CaMKII was involved in cardiomyocyte hypertrophy induced by agonists such as endothelin-1, leukemia inhibitory factor (LIF), and phenylephrine (Sei *et al.*, 1991; Ramirez *et al.*, 1997; Kato *et al.*, 2000; Zhu *et al.*, 2000). Several transgenic mouse models subsequently confirmed a role for CaMK in activation of the hypertrophic gene program and development of

hypertrophy (Table I). Transgenic mice overexpressing calmodulin were generated nearly 10 years ago and shown to develop severe cardiac hypertrophy (Gruver *et al.*, 1993). This subsequently was demonstrated to be associated with an increase in the autonomous activity of CaMKII *in vivo* (Colomer and Means, 2000). Pronounced hypertrophy also develops in transgenic mice that overexpress CaMKIV (Passier *et al.*, 2000). This is associated with specific changes in gene expression. However, CaMKIV knockout (KO) mice still are able to develop hypertrophy after TAC (Colomer *et al.*, 2003), presumably because CaMKIV is not one of the major CaMK isoforms present in the heart (Edman and Schulman, 1994; Colomer *et al.*, 2003).

Since hypertrophic growth is associated with a specific program of altered gene expression, and CaMKII is implicated in this response, we hypothesized that CaMKII isoforms expressed in the nucleus would selectively regulate hypertrophic transcriptional responses. In support of this, we reported that transient expression of the nuclear δ_B isoform of CaMKII in neonatal rat ventricular myocytes induced expression of the atrial natriuretic factor (ANF) gene, an established indicator of cardiomyocyte hypertrophy, as indicated by enhanced transcriptional activation of an ANF-luciferase reporter gene and increased ANF protein (Ramirez *et al.*, 1997). The nuclear localization signal of

TABLE I
Summary of Animal Models Showing Ca^{2+} /Calmodulin-dependent Protein Kinase (CaMK)
Involvement in Cardiac Hypertrophy

Animal model	Phenotype and effects	References
Hypertensive rat models	Cardiac hypertrophy and increased CaMKII expression	Hagemann <i>et al.</i> , 2001
Spontaneously hypertensive rats	Cardiac hypertrophy and increased CaMKII activity	Boknik <i>et al.</i> , 2001
Coronary artery ligation rabbit	Cardiac hypertrophy and increased CaMKII activity	Currie <i>et al.</i> , 1999
Transverse aortic constricted mice	Cardiac hypertrophy and increased CaMKII expression and activity	Colomer <i>et al.</i> , 2003; Zhang <i>et al.</i> , 2003
Calmodulin TG mice	Severe cardiac hypertrophy and increased CaMKII activity	Gruver <i>et al.</i> , 1993; Colomer <i>et al.</i> , 2000
CaMKIV TG mice	Cardiac hypertrophy through MEF2 activation	Passier <i>et al.</i> , 2000
CaMKII δ_B TG mice	Cardiac hypertrophy and dilated cardiomyopathy	Zhang <i>et al.</i> , 2002

Abbreviations: TG, transgenic; MEF, myocyte enhancer factor.

CaMKII δ_B was shown to be required for this response, as transient expression of CaMKII δ_C did not result in enhanced ANF expression. Indeed, CaMKII heteromultimers formed predominantly of δ_C subunits were excluded from the nucleus and failed to induce ANF expression (Ramirez *et al.*, 1997). These findings are consistent with the observation that constitutively active CaMKI and CaMKIV, which enter the nucleus, induce a hypertrophic response in cardiomyocytes *in vitro* (Passier *et al.*, 2000). We recently reported that hypertrophic growth occurs in transgenic mice that overexpress the CaMKII δ_B isoform, which is highly concentrated in cardiomyocyte nuclei (Zhang *et al.*, 2002).

B. CALCINEURIN SIGNALING IN CARDIAC HYPERTROPHY

1. Calcineurin and Transcriptional Regulation

Ca²⁺/calmodulin-dependent protein phosphatase 2B (PP2B) or calcineurin is a serine/threonine protein phosphatase that is activated by sustained elevations in intracellular Ca²⁺. The sufficiency of calcineurin to promote cardiac hypertrophy has been demonstrated *in vitro* and *in vivo* (Molkentin *et al.*, 1998; De Windt *et al.*, 2000). *In vitro*, adenoviral expression of calcineurin is sufficient to induce hypertrophy in neonatal rat ventricular myocytes (De Windt *et al.*, 2000). *In vivo*, cardiac-specific overexpression of an activated truncation mutant of calcineurin leads to profound hypertrophy that rapidly progresses to dilated heart failure by 2–3 months of age (Molkentin *et al.*, 1998). In cardiomyocytes, calcineurin dephosphorylates the nuclear factor of activated T cells (NFAT) transcription factor, which then translocates to the nucleus and interacts with the cardiac-restricted zinc finger transcription factor GATA4 to activate the gene expression of B-type natriuretic factor, a marker for cardiac hypertrophy and heart failure (Molkentin *et al.*, 1998). Another line of evidence for NFAT regulating cardiac transcriptional responses downstream of calcineurin is that transgenic (TG) mice overexpressing a constitutively nuclear NFATc4, one of the NFAT family members, develop profound hypertrophy within 2–3 months of age, whereas overexpression of full-length NFATc4 does not produce detectable hypertrophy (Molkentin *et al.*, 1998). The transcription factor MEF2 also is involved in calcineurin signals through a post-translational mechanism (Blaeser *et al.*, 2000). Two other transcription factors, NF- κ B and Elk-1, can be regulated by calcineurin (Meyer *et al.*, 1997; Tian and Karin, 1999).

2. Calcineurin Involvement in Hypertrophic Responses

A calcineurin inhibitor cyclosporin A (CsA) has been shown to attenuate the hypertrophic response induced by phenylephrine, angiotensin II, and endothelin-1 in cardiomyocytes (Molkentin *et al.*, 1998; Zhu *et al.*, 2000), suggesting that calcineurin is activated in response to these hypertrophic agonists. Increased

calcineurin activity, as well as increased calcineurin mRNA and protein expression, subsequently has been demonstrated in cardiomyocytes stimulated with phenylephrine, angiotensin II, and serum (Taigen *et al.*, 2000). Myriad studies have demonstrated that calcineurin is activated in pressure overload- and exercise-induced hypertrophy (for a review, see Molkentin, 2000). However, others have reported either no change or decreases in cardiac calcineurin activity (for a review, see Molkentin, 2000). Disparate findings also are evident in studies using systemic administration of calcineurin inhibitors, CsA, or FK506 to evaluate the role of calcineurin in cardiac hypertrophy (Olson and Williams, 2000). More-specific inhibitors of calcineurin might provide less-equivocal information. For example, Cain/Cabin-1 or A-kinase anchoring protein 79 (AKAP79) inhibited calcineurin activity and have been shown to attenuate phenylephrine and angiotensin II-induced cardiomyocyte hypertrophy *in vitro* (Taigen *et al.*, 2000) and reduced catecholamine infusion or pressure overload-induced hypertrophy *in vivo* (De Windt *et al.*, 2001). In addition, the response to hypertrophic stimuli is impaired in calcineurin-deficient mice and calcineurin dominant-negative TG mice (for a review, see Wilkins and Molkentin, 2002). Similarly, cardiac-specific overexpression of modulatory calcineurin-interacting protein 1 (MCIP1) prevents the hypertrophic response to calcineurin overexpression, isoproterenol infusion, exercise, and pressure overload (for a review, see Wilkins and Molkentin, 2002). However, studies with MCIP1 also indicate differential effects, depending on the nature of the hypertrophic stimulus. Thus, in MCIP1-null mice, the hypertrophic response to activated calcineurin overexpression was exacerbated, whereas the response to pressure overload or chronic adrenergic stimulation was blunted (Vega *et al.*, 2003).

IV. Cardiac Ca^{2+} Transients and Physiological Ca^{2+} Regulation

In the heart, intracellular Ca^{2+} transients are regulated in a beat-to-beat manner (70 beats/minute in human, > 400 beats/minute in mice), acting as transducers of excitation-contraction coupling (E-C coupling). During the cardiac action potential, Ca^{2+} enters the cell through voltage-dependent Ca^{2+} channels (L-type Ca^{2+} channel) and subsequently binds and activates the ryanodine receptor (RyR) on the SR to trigger further Ca^{2+} release (Figure 2A). This process, termed Ca^{2+} -induced Ca^{2+} release (CICR), serves to amplify and coordinate the Ca^{2+} signal. The elemental event in Ca^{2+} release from the SR is the Ca^{2+} spark. Ca^{2+} sparks occur at low frequency in diastole and in a synchronized manner, which leads to large Ca^{2+} transients and coordinated contractions during systole. To allow for muscle relaxation between contractions, cytosolic Ca^{2+} must be decreased quickly. This is accomplished by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), which removes Ca^{2+} to the extracellular space, and by the SR Ca^{2+} ATPase (SERCA), which mediates Ca^{2+} uptake into the SR.

FIG. 2. Ca^{2+} regulation in ventricular myocytes. (A) Normal physiological Ca^{2+} regulation. Ca^{2+} entering through the L-type Ca^{2+} channel (LTCC) triggers Ca^{2+} release through ryanodine-receptor (RyR). Ca^{2+} reuptake into the sarcoplasmic reticulum (SR) is mediated by the SR Ca^{2+} ATPase (SERCA), which is negatively regulated by phospholamban (PLB). The $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (NCX) can serve to remove Ca^{2+} from the cytosol. Phosphorylations (shown as ~P) of the LTCC, RyR, NCX, and PLB regulate their functions. A normal Ca^{2+} transient is illustrated by the solid line. (B) Pathophysiological changes associated with heart failure, including altered expression levels of Ca^{2+} regulatory proteins (as indicated by difference in size compared to (A)) and altered phosphorylation state (indicated by the number of ~P). The Ca^{2+} transient (solid line) is smaller and slower in failing heart (compared to normal, shown as broken line in (B)).

Two types of Ca^{2+} channels (L and T) contribute to Ca^{2+} influx. The L-type Ca^{2+} channel (LTCC) predominates in the ventricle. Ca^{2+} current through LTCC (I_{CaL}) is the most-important trigger of Ca^{2+} release. This current is regulated by voltage and cytosolic Ca^{2+} (Bers and Perez-Reyes, 1999). Stimuli that increase cAMP and activate PKA clearly enhance single-channel activity as well as whole-cell Ca^{2+} currents in cardiomyocytes (Bers, 2002). This contributes significantly to the positive inotropic effects of the β -adrenergic receptor pathway.

The RyR (RyR2 in heart) channel is a tetrameric structure comprised of four monomeric subunits, each of $\approx 565,000$ Daltons. Ca^{2+} binds to and activates the RyR to release Ca^{2+} from the SR. SR Ca^{2+} release also is regulated by SR Ca^{2+} content, with decreases in SR Ca^{2+} content facilitating closure of RyR (cessation of Ca^{2+} release) and increases in SR Ca^{2+} content increasing the Ca^{2+} sensitivity of RyR to open (Bers, 2002). The RyR has been shown to form a macromolecular complex with PKA, protein phosphatases PP1 and PP2A, FK-506 binding protein (FKBP 12.6), mAKAP, and sorcin (Meyers *et al.*, 1995; Marx *et al.*, 2000). Recently, we showed that CaMKII also associates with the RyR (Zhang *et al.*, 2003). RyR activity is regulated by its phosphorylation state, the best-described mechanism being RyR phosphorylation by PKA, which results in dissociation of FKBP12.6 from the channels and increased channel open probability (Marx *et al.*, 2000).

Ca^{2+} uptake into the SR is mediated by SERCA. SERCA plays an important role in the declining phase of the Ca^{2+} transient. The activity of SERCA2a, the isoform expressed in the heart, is regulated by intracellular Ca^{2+} concentration and PLB. PLB is an endogenous inhibitor of SERCA that is regulated by phosphorylation. PLB is phosphorylated at Ser16 by PKA (Simmerman *et al.*, 1986), which decreases its ability to inhibit SERCA activity. Phosphorylation of PLB by PKA thus accelerates Ca^{2+} uptake, contributing to the more-rapid decline of Ca^{2+} transients and contractions induced by β -adrenergic receptor stimulation (lusitropic effect). PLB is also phosphorylated at Thr 17 (Simmerman *et al.*, 1986), a site for CaMKII. The effects of this phosphorylation are more controversial.

The $\text{Na}^+/\text{Ca}^{2+}$ exchanger, NCX1, is highly expressed in the heart. Under physiological conditions, the major role of NCX is to extrude Ca^{2+} , although NCX can act in the reversed mode (Ca^{2+} influx mode), which occurs in the presence of high intracellular Na^+ and high membrane voltage (Nuss and Houser, 1992). Hilgemann and colleagues have demonstrated that PIP_2 is a positive regulator of NCX and suggested that this is the primary mechanism for ATP-dependent NCX activation (Hilgemann and Ball, 1996). Stimuli coupled to Gq and phospholipase C activation could affect the local cellular PIP_2 level. This might contribute to the regulation of NCX activity, a hypothesis that experimentally requires validation. Although regulation of NCX activity by kinases is not

established, it has been suggested that β -adrenergic agonists enhance NCX activity through PKA (Perchenet *et al.*, 2000) and that α -adrenergic agonists and phorbol ester enhance NCX activity through PKC (Iwamoto *et al.*, 1996).

V. Physiological Role of CaMK in Cardiomyocyte Ca^{2+} Handling

CaMKII has been implicated in the modulation of several key proteins involved in acute regulation of ventricular myocyte Ca^{2+} homeostasis. Published studies demonstrate that CaMKII can phosphorylate RyR (Witcher *et al.*, 1991; Hain *et al.*, 1995), SERCA (Xu *et al.*, 1993; Toyofuku *et al.*, 1994), PLB (Le Peuch *et al.*, 1979; Simmerman *et al.*, 1986), and the LTCC or an associated regulatory protein (Dzhura *et al.*, 2000). Thus, CaMKII has the potential to significantly affect acute Ca^{2+} regulation and E-C coupling in cardiomyocytes (Figure 3).

Studies using CaMKII inhibitors in isolated cardiomyocytes suggest that CaMKII is the mediator of Ca^{2+} current (I_{Ca}) facilitation (Anderson *et al.*, 1994; Xiao *et al.*, 1994; Yuan and Bers, 1994), although the site of CaMKII action is

FIG. 3. Regulation of cardiomyocyte Ca^{2+} homeostasis by CaMKII. CaMKII can phosphorylate the RyR2 at Ser2809 (and others), SERCA2a at Ser38, PLB at Thr17, and the LTCC or an associated regulatory protein (the actual site is unknown). Most of these phosphorylations have functional consequences, indicating that CaMKII could profoundly affect Ca^{2+} handling in cardiomyocytes.

not clear. It is likely that CaMKII phosphorylates the L-type Ca^{2+} channel complex or an associated regulatory protein, since CaMKII colocalizes with LTCC in myocytes (Xiao *et al.*, 1994) and CaMKII can activate LTCC when it is applied to the cytoplasmic face of excised cell membrane patches (Dzhura *et al.*, 2000). It has been reported that CaMKII is involved in the development of early after-depolarizations (EADs) and arrhythmias as a consequence of LTCC activation *in vitro* and *in vivo* (Anderson *et al.*, 1998; Wu *et al.*, 1999, 2002).

CaMKII has been reported to phosphorylate the same site (Ser2809) as PKA on the RyR2 (Witcher *et al.*, 1991; Marx *et al.*, 2000; Rodriguez *et al.*, 2003). However, the functional consequence of CaMKII-mediated phosphorylation is not yet clear. RyR2 phosphorylation by CaMKII has been suggested to alter RyR2 gating properties. While some studies indicate that CaMKII increases RyR2 open probability (Witcher *et al.*, 1991; Hain *et al.*, 1995), others find that CaMKII decreases RyR2 open probability (Lokuta *et al.*, 1995). To assess the intrinsic effect of CaMKII on E-C coupling, Bers' laboratory used voltage-clamped ventricular myocytes and demonstrated that, for a given SR Ca^{2+} load and I_{Ca} trigger, inhibition of CaMKII by KN-93 diminished the Ca^{2+} -dependent increase in SR Ca^{2+} release (relative to total SR) (Li *et al.*, 1997). This finding supports the hypothesis that activation of CaMKII by Ca^{2+} transients phosphorylates RyR2 and enhances the efficacy of E-C coupling in cardiomyocytes. Whether CaMKII phosphorylation of RyR2 can dissociate FKBP12.6, as observed for PKA, is still not clear. An important recent finding from Colyer's laboratory is that CaMKII appears to phosphorylate at least four sites, in addition to Ser2809, on RyR2 *in vitro* (Rodriguez *et al.*, 2003). Whether this occurs *in vivo* and how this alters E-C coupling have not been established.

As previously discussed, phosphorylation of PLB plays a major role in regulating cardiac SERCA activity. PLB can be phosphorylated by CaMKII at Thr17 (Le Peuch *et al.*, 1979; Simmerman *et al.*, 1986), a site adjacent to the PKA phosphorylation site (Ser16). Phosphorylation of PLB relieves its inhibitory effects on SERCA activity, thereby accelerating Ca^{2+} transport. The functional role of dual-site PLB phosphorylation has been examined extensively over the past decade. Several studies have suggested that PLB phosphorylation at Ser16, the PKA site, is a prerequisite for PLB phosphorylation at Thr17 *in vivo* (Luo *et al.*, 1998) and is sufficient to mediate the maximal contractile effects of β -adrenergic stimulation (Chu *et al.*, 2000). However, emerging evidence has shown that Thr17 phosphorylation of PLB by CaMKII can occur independently of Ser16 phosphorylation. First, it has been demonstrated in genetically targeted mice that mutation of Ser16 in PLB (S16A) does not prevent Thr17 phosphorylation (Chu *et al.*, 2000). Second, it has been reported that electrical pacing of rat ventricular myocytes increases CaMKII-dependent phosphorylation of PLB at Thr17 in a frequency-dependent manner, without altering Ser16 phosphorylation (Hagemann *et al.*, 2000). Third, it has been reported that an increase in Thr17

phosphorylation can be produced by an increase in intracellular Ca^{2+} (with a simultaneous inhibition of phosphatases or acidosis) in the absence of β -adrenoceptor stimulation (Mundina-Weilenmann *et al.*, 1996; Vittone *et al.*, 1998). Finally, in CaMKII δ_{C} TG mice, PLB phosphorylation at the CaMKII site was increased without increased phosphorylation of the PKA site (Zhang *et al.*, 2003). These observations all indicate that PLB phosphorylation by CaMKII can occur independently of and serve a function distinct from that of PLB phosphorylation by PKA under physiological or pathophysiological conditions. Of particular interest, recent studies using PLB site-specific mutant mice have shown that both PLB phosphorylation sites are involved in the mechanical recovery after ischemia, with Thr17 appearing to play the major role (Said *et al.*, 2003). In addition, it recently was reported that targeted inhibition of CaMKII in cardiac longitudinal SR transgenic mice causes selective decreases in PLB phosphorylation at Thr17 but not at Ser16 and results in cardiac dysfunction (Ji *et al.*, 2003).

Frequency-dependent acceleration of relaxation (FDAR) is an important intrinsic physiological mechanism that contributes to faster relaxation and diastolic filling as heart rate increases. Published studies have suggested that FDAR is mediated by CaMKII rather than PKA (Bassani *et al.*, 1995) and might be due to enhanced SR Ca^{2+} uptake secondary to CaMKII-mediated PLB phosphorylation (Schouten, 1990). However, while PLB would appear to be a likely target for CaMKII effects on FDAR, this response is still prominent in PLB KO mice. Moreover, it can be inhibited by the CaMKII inhibitor, KN-93 (DeSantiago *et al.*, 2002). Thus, targets of CaMKII other than PLB must contribute to FDAR.

The question of whether SERCA function is regulated directly by CaMKII phosphorylation remains controversial. Some investigators have shown that CaMKII can directly phosphorylate cardiac SERCA (SERCA2a) on Ser38, resulting in increases in its enzymatic activity and therefore the maximal velocity (V_{max}) of Ca^{2+} transport (Xu *et al.*, 1993; Toyofuku *et al.*, 1994). However, studies from other groups present contradictory findings (Odermatt *et al.*, 1996; Reddy *et al.*, 1996). One study failed to observe phosphorylation of SERCA2a by CaMKII (Reddy *et al.*, 1996) and another observed CaMKII-mediated phosphorylation but did not observe stimulation of SERCA2a activity (Odermatt *et al.*, 1996). Thus, the physiological role of SERCA2a phosphorylation by CaMKII remains unclear.

VI. Pathophysiological Changes of Ca^{2+} in the Heart

A variety of stresses are imposed upon the heart by pathophysiological conditions, including high blood pressure, ischemia, infarction, and virus infection. Since cardiomyocytes are terminally differentiated and have lost their ability to proliferate, they adapt by increasing cell size, resulting in myocyte hypertrophy and cardiac enlargement. Although this is a compensatory response,

it also serves as an independent risk factor for development of heart failure, which is characterized by the heart's inability to generate sufficient force for blood ejection. The failing heart exhibits not only structural changes but also marked alterations in Ca^{2+} regulatory protein expression and phosphorylation. Ca^{2+} transients in ventricular myocytes isolated from failing hearts are generally of reduced amplitude and characterized by slower times to peak and to decline. The diastolic level of Ca^{2+} is higher in failing vs. normal heart. Changes in expression levels and activity of several Ca^{2+} regulatory proteins are thought to underlie the dysregulation of Ca^{2+} transients in heart failure (Figure 2B). Diminished Ca^{2+} transients are major determinants of the decreased cellular contractile function. Indeed, Ca^{2+} dysregulation, with resultant changes in E-C coupling, has been suggested as a causal mechanism of heart failure. Ca^{2+} dysregulation may contribute to cardiomyocyte apoptosis (e.g., via mitochondrial Ca^{2+} overloading or calpain activation), leading to progressive loss of cardiomyocytes. Myocyte cell death is not compensated by myocyte cell replacement and, accordingly, myocyte loss is considered to contribute to the development of heart failure.

Furthermore, less Ca^{2+} release is induced by a given Ca^{2+} current in heart failure, a phenomenon referred to as decreased E-C coupling gain (Gomez *et al.*, 2001). Decreased RyR expression, observed in numerous models of heart failure, could contribute to this. However, as described earlier, RyR function is also highly regulated by its phosphorylation. RyR hyperphosphorylation is observed in heart failure (Marks, 2000; Marx *et al.*, 2000). RyR hyperphosphorylation results in the dissociation of FKBP12.6 from RyR, enhancing Ca^{2+} leakage during diastole and thereby decreasing the pool of Ca^{2+} available for release during the contractile cycle (Marks, 2000; Reiken *et al.*, 2003a). Therefore, RyR hyperphosphorylation has been suggested to contribute to the decrease in E-C coupling gain in heart failure through the decrease in SR Ca^{2+} loading. The maladaptive effects of RyR hyperphosphorylation may be one reason that chronic β blockade improves cardiac contractility and prolongs survival in patients with heart failure. Indeed, recent data indicate that β blockade normalizes the phosphorylation status of RyR, resulting in RyR stabilization and decreased Ca^{2+} leakage from the SR in both animal models and failing human heart (Doi *et al.*, 2002; Reiken *et al.*, 2003b). In addition to this hypothesized mechanism, spatial remodeling of T tubules (decreased number of T tubules) and/or the dyad (i.e., distance between LTCC and RyR) have been postulated as mechanisms for the decreased E-C coupling gain (Gomez *et al.*, 2001).

No changes in peak Ca^{2+} currents are observed in most studies of heart failure (Benitah *et al.*, 2002), although channel density has been suggested to be reduced (He *et al.*, 2001). Increased open probability of LTCC, perhaps as a result of increased basal phosphorylation, might compensate for the decreased channel density (Schroder *et al.*, 1998; X. Chen *et al.*, 2002). The functional

contribution of changes in Ca^{2+} currents to heart failure requires further investigation.

SR Ca^{2+} uptake is decreased in many animal models of heart failure. This defect, like increased diastolic Ca^{2+} leak through phosphorylated RyR, would diminish the releasable Ca^{2+} pool and thus contribute to impaired contractile function. Decreases in SR Ca^{2+} uptake also contribute to the prolonged Ca^{2+} transients observed in cardiomyocytes from failing heart. A mechanistic basis for the decreased Ca^{2+} uptake is diminished SERCA protein expression, observed in some, but not all, experimental models of heart failure (Movsesian *et al.*, 1994; Schwinger *et al.*, 1995). In support of a pathophysiological role for decreased SERCA expression, recent evidence indicates that adenoviral SERCA gene delivery improves cellular contractile function and Ca^{2+} transients in human heart failure (Del Monte *et al.*, 2002).

PLB generally is not decreased to the same extent as SERCA in heart failure; thus, there is a lower ratio of SERCA/PLB and enhanced SERCA inhibition in heart failure (Houser *et al.*, 2000). PLB phosphorylation also appears to be decreased (Huang *et al.*, 1999; Houser *et al.*, 2000), providing another mechanism for enhanced SERCA inhibition in heart failure. Interestingly, recent evidence indicates that a mutation in PLB can, by itself, lead to human heart failure (Schmitt *et al.*, 2003). Development of PLB inhibitors therefore has been touted as a promising therapeutic strategy to improve SR Ca^{2+} content and other cellular defects in heart failure (Chien *et al.*, 2003).

The role of NCX in removing cytosolic Ca^{2+} gains importance in the face of decreased SR Ca^{2+} uptake in heart failure. In many, but not all, cases (for a review, see Sipido *et al.*, 2002), NCX is upregulated in heart failure. This is thought to be a compensatory response to prevent Ca^{2+} overloading. Recently, it has been reported that NCX is hyperphosphorylated by PKA, resulting in increased activity of the exchanger in failing heart (Wei *et al.*, 2003). While increased NCX activity initially would be adaptive, excessive or sustained NCX activation could further contribute to decreased SR Ca^{2+} content by removing cytosolic Ca^{2+} , diminishing systolic Ca^{2+} transients and contractile function.

VII. Pathophysiological Role of CaMK in the Heart

A. ROLE OF CAMK IN THE DEVELOPMENT OF HEART FAILURE

Changes in CaMKII have been associated with development of heart failure. It has been reported that CaMKII activity is increased ≈ 3 -fold and that CaMKII expression is increased ≈ 2 -fold in human failing hearts with dilated cardiomyopathy (Hoch *et al.*, 1999; Kirchhefer *et al.*, 1999). Conversely, in a rat heart failure model induced by myocardial infarction (Netticadan *et al.*, 2000) and in

a canine model of heart failure produced by intracoronary microembolization (Mishra *et al.*, 2003), CaMKII activity and expression are reduced. We and others observed that, in the TAC-induced hypertrophy mouse model, there is not only acute CaMKII activation but also an increase in CaMKII δ_C expression that is sustained for at least 1 week (Colomer *et al.*, 2003; Zhang *et al.*, 2003). Upregulation and activation of CaMKII have been correlated with changes in cardiac function (cardiac index and ejection fraction) in patients (Hoch *et al.*, 1999; Kirchhefer *et al.*, 1999). Sustained CaMKII activation could play a causal role in the development of heart failure. However, whether changes in CaMKII are causal or secondary to the development of cardiac diseases remains to be established. Nonetheless, the findings clearly indicate that regulatory changes in CaMKII expression occur in association with cardiac pathology.

B. EFFECTS OF CaMKII δ_C OVEREXPRESSION ON Ca²⁺ HANDLING IN TRANSGENIC MICE

As was discussed, CaMKII plays important roles in Ca²⁺ cycling and E-C coupling in cardiomyocytes *in vitro*, although the extent to which this can occur *in vivo* has not been addressed. Accordingly, our laboratory generated TG mice that expressed the cytoplasmic CaMKII δ_C isoform in the heart and examined phosphorylation of Ca²⁺ regulatory proteins and concomitant changes in cellular Ca²⁺ regulation. These mice developed a dilated cardiomyopathy characterized by a significant decrease in cardiac function and premature death (Zhang *et al.*, 2003). The time course for development of functional impairment and extent of lethality were related to the gene dosage for CaMKII expression in several TG founder lines (Zhang *et al.*, 2003). Further studies exploring the mechanisms by which CaMKII expression induced these phenotypic changes examined changes in the phosphorylation state of Ca²⁺ regulatory proteins in cardiac homogenates and alterations in Ca²⁺-handling properties in adult cardiomyocytes isolated from the TG mice (Maier *et al.*, 2003; Zhang *et al.*, 2003).

We hypothesized that the initial effects of CaMKII δ_C on Ca²⁺ handling would be adaptive responses that serve to increase contractile function. Our finding of a rapid increase in CaMKII activation in response to pressure overload induced by TAC (Zhang *et al.*, 2003) is consistent with CaMKII serving such an adaptive role. Enhanced PLB phosphorylation, which would be expected to increase SR Ca²⁺ transport and SR Ca²⁺ stores, was evidenced by the increased PLB phosphorylation at Thr17 site in CaMKII δ_C TG mouse hearts vs. wild type (WT) (Zhang *et al.*, 2003). We also demonstrated that RyR2 phosphorylation was increased in CaMKII δ_C TG vs. WT mouse hearts. This was established both by back phosphorylation (Zhang *et al.*, 2003) and through use of a phospho-specific antibody for Ser2809 that showed a significant increase in phospho-RyR2 in TG vs. WT hearts (T. Zhang and J.H. Brown, unpublished data).

As discussed earlier, hyperphosphorylation of RyR2 by PKA is suggested to play a major role in the etiology of heart failure (Marx *et al.*, 2000). We hypothesized that this also might occur with CaMKII-mediated changes in RyR2 phosphorylation and function. The functional consequences of RyR2 phosphorylation were assessed in myocytes isolated from the CaMKII δ_C TG mice (Maier *et al.*, 2003). Diastolic Ca²⁺ spark frequency was increased in TG myocytes and the sparks were of increased width and prolonged duration. The overall diastolic SR Ca²⁺ leakage was 4.3-fold higher in TG vs. WT cardiomyocytes. Importantly, SR Ca²⁺ release during twitch (relative to the total SR Ca²⁺ pool) was found to be increased in TG vs. WT, despite lower SR Ca²⁺ load and diastolic intracellular Ca²⁺. Another important finding was that RyR2 phosphorylation and Ca²⁺ spark frequency were increased at early stages, prior to development of failure (assessed by echocardiography), in CaMKII δ_C TG mouse hearts. We also reported that the increased Ca²⁺ spark frequency in isolated TG myocytes was normalized by acute CaMKII inhibition and that CaMKII δ was associated with the RyR2 in immunoprecipitation studies (Zhang *et al.*, 2003). Taken together, these data demonstrate that CaMKII mediates RyR2 phosphorylation *in vivo*, that this results in increases in SR Ca²⁺ spark frequency, and that these precede (and could therefore be causal in) the development of failure (Zhang *et al.*, 2003).

Other changes in Ca²⁺ handling were observed in CaMKII δ_C TG mice, including direct effects of overexpressed CaMKII δ_C on peak I_{Ca} and FDAR (Maier *et al.*, 2003). Peak I_{Ca} was slightly increased in TG vs. WT and was acutely reversed by CaMKII inhibition. However, CaMKII-dependent I_{Ca} facilitation still was present in TG mice, suggesting that CaMKII activation in the TG mouse hearts was not sufficient to saturate I_{Ca} facilitation. FDAR was only slightly (albeit significantly) enhanced in TG mice (Maier *et al.*, 2003), implying that CaMKII is involved in FDAR but that endogenous CaMKII levels in WT mice may not be rate limiting. Changes in SERCA (decreased) and NCX (increased) expression and function observed in CaMKII TG mice are presumably secondary to heart failure development in these mice, since there is no evidence that CaMKII can directly downregulate SERCA2a or upregulate NCX function. Similar changes in SERCA2a and NCX function are seen in numerous heart failure models.

VIII. Ca²⁺ and Apoptosis

A. IMPORTANCE OF APOPTOSIS IN ISCHEMIA/REPERFUSION INJURY AND THE DEVELOPMENT OF HEART FAILURE

Apoptosis or programmed cell death plays an important role in development and has been implicated in diseases, including cancer, autoimmune diseases, and

degenerative disorders. Apoptosis is widely recognized as a factor contributing to heart failure. Over the last decade, it has been shown that apoptotic cell death is associated with myocardial infarction, ischemia/reperfusion, and cardiomyopathy (Gottlieb *et al.*, 1994; Olivetti *et al.*, 1997). The development of apoptosis is highly regulated in a step-by-step fashion by many signaling events, providing the potential for multiple sites of therapeutic intervention. There are two relatively distinct pathways in apoptosis: the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway. Both eventually converge on caspase-3, a key component of the apoptotic machinery.

In response to ischemia/reperfusion, both apoptosis and necrosis are observed and the contribution of each to cardiomyocyte cell death is controversial. However, it has been reported that caspase inhibitors, which block apoptosis, protect against ischemia/reperfusion damage (Yaoita *et al.*, 1998). A mitochondrial permeability transition pore inhibitor has been shown to be protective against ischemia/reperfusion in ventricular myocytes and isolated hearts (Griffiths and Halestrap, 1993; Murata *et al.*, 2001). These findings implicate apoptosis in the pathological response to ischemia/reperfusion. Interestingly, while the border zone of infarction shows apoptotic cell death, infarct zones in the myocardium show necrosis, raising the possibility that cell death starts by way of the more-tidy apoptotic pathway but ultimately regresses to one with necrotic features (Yaoita *et al.*, 1998).

Myocyte loss by apoptosis is recognized as a major contributing factor in the transition from compensated (adaptive) hypertrophy to heart failure (maladaptation). Conditional KO of gp130, a receptor through which cytokines regulate cardiomyocyte survival, has been reported to result in massive induction of myocyte apoptosis and rapid onset of dilated cardiomyopathy in response to aortic banding (Hirota *et al.*, 1999). Direct induction of apoptosis by conditional caspase activation also induces heart failure in transgenic mice (Wencker *et al.*, 2003). Activation of the $G_{\alpha q}$ signaling pathway, when sustained or exacerbated, induces heart failure with apoptosis (Adams *et al.*, 1998,2000). The peripartum cardiomyopathy seen in $G_{\alpha q}$ transgenic mice is markedly attenuated by caspase inhibition (Hayakawa *et al.*, 2003).

B. Ca^{2+} , PERMEABILITY TRANSITION PORE, AND CARDIOPROTECTION

Many experimental models of cardiomyocyte apoptosis — including ischemia/reperfusion and sustained $G_{\alpha q}$ signaling — are associated with mitochondrial changes due to the opening of the mitochondrial permeability transition pore (PT pore) (Halestrap *et al.*, 1997; Adams *et al.*, 2000). The PT pore is considered to be a megachannel with permeability to ions and solutes up to ≈ 1500 daltons. Although not all of the molecules comprising the PT pore are

known, the adenine nucleotide translocator (ANT) and voltage-dependent anion channel (VDAC) are among them. Long-lasting PT pore opening has been reported to dissipate the mitochondrial membrane potential and decrease ATP production (Kroemer and Reed, 2000). Cytochrome *c* is released through the PT pore and acts, along with other cytosolic factors, to promote caspase-9 activation. Subsequent caspase-3 activation mediates DNA cleavage into nucleosomal fragments. PT pore opening has been reported to be regulated by several factors. Increases in Ca^{2+} , reactive oxygen species (ROS), and proapoptotic Bcl-2 family proteins (e.g., Bad, Bax, tBid) induce PT pore opening, while antiapoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-xl inhibit the PT pore (for a review, see Kroemer and Reed, 2000).

The involvement of Ca^{2+} in regulating mitochondrial function has been appreciated for many years. Mitochondria take up cytosolic Ca^{2+} via a Ca^{2+} uniporter. Recent studies show that mitochondrial Ca^{2+} changes in a beat-to-beat manner in cardiomyocytes (Robert *et al.*, 2001). Furthermore, a close relationship between the SR and mitochondria in cardiomyocytes is illustrated by studies demonstrating that Ca^{2+} derived from SR (via Ca^{2+} -induced Ca^{2+} release mechanism) is efficiently transmitted to the mitochondria (Szalai *et al.*, 2000). A more-recent focus has been on the role of mitochondrial Ca^{2+} in PT pore opening (Crompton, 1999; Murata *et al.*, 2001). Experiments using isolated mitochondria or permeabilized cells clearly demonstrate that high concentration of Ca^{2+} can induce PT pore opening (Crompton, 1999). Our unpublished observations demonstrate that ionomycin, a Ca^{2+} ionophore, induces PT pore opening (assessed by loss of mitochondrial membrane potential) and induces DNA laddering in neonatal rat ventricular myocytes. We also have observed that cytosolic Ca^{2+} dysregulation and resultant Ca^{2+} overloading directly induce PT pore opening and apoptosis in cardiomyocytes subject to sustained stimulation of G α_q signaling pathways, while chelation of Ca^{2+} with EGTA (ethylene glycol-bis(beta-amino ethyl ether)-N, N, N', N'-tetraacetic acid) inhibits these responses (S. Miyamoto and J. H. Brown, unpublished data).

Another mitochondrial channel that may be involved in regulating mitochondrial Ca^{2+} concentration and PT pore function is the mitochondrial ATP-sensitive potassium channel (mitoK_{ATP}). MitoK_{ATP} has attracted considerable interest since Liu and coworkers (1998) reported that diazoxide selectively opens these channels and confers cardiac protection against subsequent ischemia. Although not all of the operative cardioprotective mechanisms have been elucidated, it has been shown that diazoxide treatment inhibits mitochondrial Ca^{2+} overloading (Murata *et al.*, 2001). Three mechanisms by which mitoK_{ATP} channel opening could lead to protection have been suggested. One is that K^+ influx reduces the driving force for Ca^{2+} by mitochondrial membrane depolarization (Holmuhamedov *et al.*, 1999). Second, opening the mitoK_{ATP} channel induces moderate production of ROS, which contributes to cardioprotection by

stimulating protein kinases, in particular, PKC (Forbes *et al.*, 2001). Third, it has been proposed that treatment with diazoxide results in release of mitochondrial Ca^{2+} by inducing flickering PT pore opening (low-conductance state) (Katoh *et al.*, 2002).

Recently, another Ca^{2+} -dependent pathway has been suggested to be responsible for cytochrome *c* release during ischemia/reperfusion (M. Chen *et al.*, 2001, 2002). Calpain, a Ca^{2+} -dependent cysteine protease, is activated by the rapid influx of Ca^{2+} stimulated by reperfusion and cleaves Bid, a proapoptotic BH3-only Bcl-2 family member. The product of the cleavage, tBid, induces mitochondrial dysfunction. Calpain has been recognized as an important mediator of necrotic cell death through proteolysis; thus, these results suggest possible cross-talk between apoptotic and necrotic pathways.

C. CAMK INVOLVEMENT IN APOPTOSIS

There is limited but compelling evidence that CaMKII can mediate signal transduction in apoptosis. Selective inhibitors of CaMKII significantly inhibit apoptotic responses induced by tumor necrosis factor alpha ($\text{TNF}\alpha$), ultraviolet irradiation, and the natural toxin microcystin (Wright *et al.*, 1997; Fladmark *et al.*, 2002). In addition, overexpression of active forms of CaMKII can induce apoptosis (Fladmark *et al.*, 2002). CaMKII has been shown to regulate expression and phosphorylation of c-FLIP (cellular Fas-associated death domain-like interleukin-1 β -converting enzyme inhibitory protein), thus modulating Fas-mediated signaling (Yang *et al.*, 2003). Our recent collaborative studies demonstrated that apoptosis induced by β 1-adrenergic receptor stimulation in adult mouse cardiomyocytes occurs through activation of CaMKII rather than PKA (Zhu *et al.*, 2003). We reported that CaMKII inhibition protected cardiomyocytes against apoptosis and that expression of the cytoplasmic CaMKII δ_C (but not the nuclear CaMKII δ_B) enhanced the apoptotic response (Zhu *et al.*, 2003). Further investigation is needed to elucidate the downstream targets of CaMKII in apoptotic signaling pathways. However, it is intriguing to consider that chronic activation or increased expression of CaMKII δ_C not only contributes to the altered Ca^{2+} handling and contractile dysfunction associated with heart failure but also serves as a mediator of apoptotic loss of cardiomyocytes.

IX. Summary

Changes in intracellular Ca^{2+} underlie multiple cardiomyocyte responses and are critical to the fundamental function of the heart as a pump. Studies carried out over the past decade demonstrate that CaMK is a ubiquitous transducer of Ca^{2+} signals. Thus, the possible role of CaMKII in cardiac function has generated considerable interest. The discovery of distinct CaMKII δ splice

variants that localize to the cytoplasm vs. the nucleus suggests that there could be precise spatial regulation of CaMKII activation and function in cardiomyocytes. Whether the cardiac CaMKII isoforms are differentially regulated by Ca^{2+} mobilized in response to particular stimuli or arising from distinct sources remains to be determined. Data presented herein suggest that the isoforms may play distinct roles in transcriptional regulation, Ca^{2+} homeostasis, and control of apoptosis. Information on dynamic changes in CaMKII isoform activation during the cardiac contraction cycle and on more-chronic changes in CaMKII activation and expression in adaptive and maladaptive phases of cardiac diseases will be necessary to gain further insight into physiological and pathological functions of CaMKII. It is abundantly clear, however, that there are multiple avenues for manipulating cardiac Ca^{2+} /CaMKII signaling pathways that hold considerable therapeutic potential.

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