Activation of CaMKII\(_{\delta C}\) Is a Common Intermediate of Diverse Death Stimuli-induced Heart Muscle Cell Apoptosis*

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Ca\(^{2+}\)-calmodulin-dependent protein kinase II (CaMKII) is expressed in many mammalian cells, with the \(\delta\) isoform predominantly expressed in cardiomyocytes. Previous studies have shown that inhibition of CaMKII protects cardiomyocytes against \(\beta_1\)-adrenergic receptor-mediated apoptosis. However, it is unclear whether activation of CaMKII is sufficient to cause cardiomyocyte apoptosis and whether CaMKII signaling is important in heart muscle cell apoptosis mediated by other stimuli. Here, we specifically enhanced or suppressed CaMKII activity using adenoviral gene transfer of constitutively active (CA-CaMKII\(_{\delta C}\)) or dominant negative (DN-CaMKII\(_{\delta C}\)) mutants of CaMKII\(_{\delta C}\) in cultured adult rat cardiomyocytes. Expression of CA-CaMKII\(_{\delta C}\) promoted cardiomyocyte apoptosis that was associated with increased mitochondrial cytochrome \(c\) release and attenuated by co-expression of Bcl-X\(_L\). Importantly, isoform-specific suppression of CaMKII\(_{\delta C}\) with the DN-CaMKII\(_{\delta C}\) mutant similar to nonselective CaMKII inhibition by the pharmacological inhibitors (KN-93 or AIP) not only prevented CA-CaMKII\(_{\delta C}\)-mediated apoptosis but also protected cells from multiple death-inducing stimuli. Thus, activation of CaMKII\(_{\delta C}\) constitutes a common intermediate by which various death-inducing stimuli trigger cardiomyocyte apoptosis via the primary mitochondrial death pathway.

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\(^2\)The abbreviations used are: CaMKII, \(\mathrm{Ca}^{2+}\)-calmodulin-dependent protein kinase II; AR, adrenergic receptor; CA, constitutively active; DN, dominant negative; SR, sarcoplasmic reticulum; PLB, phospholamban; HA, hemagglutinin; WT, wild type; m.o.i., multiplicity of infection; \(\beta\)-gal, \(\beta\)-galactosidase; TUNEL, deoxynucleotidyltransferase-mediated dUTP nick end labeling.
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CaMKII activity to cause cell death. Because apoptosis is a key cause factor of chronic heart failure, the identification of fundamental molecular players involved in heart muscle cell loss has become, in the past decade, an important research focus in the field of cardiovascular biology and medicine.

The goals of the present study were (a) to determine whether activation of the cardiac cytosolic isoform, CaMKII<sub>SC</sub>, is sufficient to trigger cardiac myocyte apoptosis and, if so, (b) to determine whether CaMKII<sub>SC</sub> is a necessary intermediate in the death signaling caused by stimuli other than chronic stimulation of β<sub>1</sub> ARs. To address these questions, we used cultured adult rat cardiac myocytes in conjunction with adenoviral gene transfer of constitutively active (CA-CaMKII<sub>SC</sub>) or dominant negative (DN-CaMKII<sub>SC</sub>) mutants of CaMKII-δC to elevate or suppress CaMKII<sub>SC</sub> activity, respectively. We demonstrate that enhanced activation of CaMKII<sub>SC</sub> is sufficient to trigger robust cardiac myocyte apoptosis via activating the mitochondrial apoptotic pathway and that inhibition of CaMKII protects cardiomyocytes not only from β<sub>1</sub> AR-induced apoptosis, as previously reported (25), but from a number of other death-inducing stimuli as well. These findings demonstrate that activation of CaMKII<sub>SC</sub> is an important common intermediate in the death signaling pathway of many different stimuli that induce apoptosis in heart muscle cells.

**EXPERIMENTAL PROCEDURES**

Construction of Viral Vectors—HA-tagged constitutively active CaMKIIδC (CA-CaMKII) was generated by replacing the residue Thr<sup>287</sup> with Asp (T287D) using the transformer site CaMK vectors encoding rat WT-CaMKII mediated gene transfer was implemented by adding adenoviral mycin, and 25 mmol/liter HEPES, pH 7.4, at 37 °C. Adenovirus-0.1% insulin-transferrin-selenium-X, 1% penicillin and streptomycin, and 25 mmol/liter HEPES, pH 7.4, at 37 °C. Adenovirus expressing Bcl-XL was obtained from the University of Pittsburgh NHLBI, National Institutes of Health Pre-clinical Vector Core.

Cell Culture and Adenoviral Gene Transfer—Single cardiac myocytes were isolated from the hearts of 2–3-month-old Sprague-Dawley rats using a standard enzymatic technique, then cultured, and infected with adenoviral vectors at a multiplicity of infection (m.o.i) indicated, as described previously (27, 28). Briefly, myocytes were plated at a density of 0.5 to 1 × 10<sup>4</sup>/cm<sup>2</sup> on coverslips or in dishes precoated with 10 µg/ml laminin. The culture medium was M199 (Sigma) plus 5 mmol/liter creatine, 2 mmol/liter 1-carnitine, 5 mmol/liter taurine, 0.1% insulin-transferrin-selenium-X, 1% penicillin and streptomycin, and 25 mmol/liter HEPES, pH 7.4, at 37 °C. Adenovirus-mediated gene transfer was implemented by adding adenoviral vectors encoding rat WT-CaMKII<sub>SC</sub>, DN-CaMKII<sub>SC</sub>, CA-CaMKII<sub>SC</sub>, Bcl-X<sub>L</sub>, or β-gal into the culture dish. The experiments were done with cells cultured 24 h after infection, unless specified otherwise.

Intracellular Acidosis and H<sub>2</sub>O<sub>2</sub> Treatment—Intracellular acidosis was induced by incubating cardiomyocytes with an acidic HEPES buffer with [pH]<sub>i</sub> of 6.6 (29). Specifically, cultured adult rat cardiomyocytes were incubated with the acidic HEPES buffer (137 mm NaCl, 4.9 mm KCl, 1.2 mm Na<sub>2</sub>PO<sub>4</sub>, 15 mm glucose, 1.2 mm MgCl<sub>2</sub>, 20 mm HEPES, 1.8 mm CaCl<sub>2</sub>, pH 5.5, for 24 h, in the presence or absence of 30-min pretreatment of cells with a CaMKII inhibitor, either KN-93 (2 µM) or AIP (5 µM), or expression of DN-CaMKII<sub>SC</sub>. In another subset of experiments, cultured cardiac myocytes were insulted with H<sub>2</sub>O<sub>2</sub> at 12 or 20 µM as indicated for 24 h in the presence or absence of 30-min pretreatment with KN-93 (2 µM) or expression of DN-CaMKII<sub>SC</sub>.

Cell Apoptosis—Cell apoptosis was detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay as previously described (25). The percentage TUNEL-positive cells was determined by randomly counting 500–800 cardiac myocytes over 20 randomly chosen fields in each culture dish. DNA fragmentation was also visualized by DNA laddering assay, as previously described (25).

Western Blotting—CaMKII-dependent phosphorylation of PLB at Thr<sup>14</sup> (PLB-Thr<sup>14</sup>) was detected by Western blot using a site-specific antibody (Badrilla, West Yorkville, UK) (15, 27). To quantify the expression of WT and mutant CaMKII<sub>SC</sub> cell lysate (30–50 µg of protein) were loaded in a Ca<sup>2+</sup>-free loading buffer containing 20 mm EDTA and immunoblotted using anti-HA (1:1000, Covance) or anti-CaMKII antibody (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Following incubation with a peroxidase-conjugated antibody, the films were exposed to the chemiluminescence (ECL; Amersham Biosciences) reaction and quantified with a video documentation system (Bio-Rad). The actin amount used as protein loading control was detected with an anti-actin antibody (Santa Cruz).

Assay of CaMKII Activity—CaMKII activity was measured, as described (27). Briefly, cell lysate (300 µg of protein) was first immunoprecipitated with anti-CaMKII antibody (1:100) in a Ca<sup>2+</sup>-free medium containing 20 mmol/liter Tris-HCl, 150 mmol/liter NaCl, 1 mmol/liter Na<sub>2</sub>EDTA, 1 mmol/liter EGTA, 1% Triton, 2.5 mmol/liter sodium pyrophosphate, 1 mmol/liter β-gal, 1 mmol/liter Na<sub>2</sub>VO<sub>4</sub>, and 1 µg/ml leupeptin, pH 7.4. The precipitated proteins were then incubated with a specific peptide substrate (KKALRRQETVDAL) to evaluate the kinase activity by <sup>32</sup>P incorporation into the substrate according to the manufacturer’s recommendations (Upstate Biotechnology Inc.), as previously described (27).

Subcellular Fractionation and Western Blot of Cytochrome c—Subcellular fractionation was performed using a previously described method (30). After centrifugation (15,000 rpm, 10 min at 4 °C), the supernatant and mitochondrial pellets were used for Western blot analysis. Anti-cytochrome c (0.5 µg/ml, BD Biosciences), or anti-RhoGDI (Abcam) (as a cytosolic marker), anti-cytochrome oxidase subunit IV (COXIV) (as a mitochondrial marker) (0.1 µg/ml) (BD Bioscience) monoclonal antibodies were utilized.

Materials—Ionomycin, thapsigargin, and isoproterenol were purchased from Sigma-Aldrich, whereas KN-93 and AIP was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA).
Statistical Analysis—The results are presented as the means ± S.E. Statistical significance was determined by one-way analysis of variance or unpaired Student’s t test when appropriate. A p value of <0.05 was considered to be statistically significant.

RESULTS

Manipulation of CaMKII<sub>SC</sub> Activity in Cultured Adult Rat Cardiomyocytes—Previous studies have shown that CaMKII activation has been implicated in β<sub>1</sub>AR-induced apoptosis of adult mouse cardiomyocytes (25) and Ca<sup>2+</sup>- influx-mediated apoptosis of feline cardiomyocytes (31). Here, we sought to determine whether there is a causal relation between the activation of CaMKII<sub>SC</sub> and heart muscle cell apoptosis. We specifically enhanced or inhibited CaMKII<sub>SC</sub> activity by adenoviral gene transfer of a constitutively active (CA-CaMKII<sub>SC</sub>) or a dominant negative CaMKII<sub>SC</sub> mutant (DN-CaMKII<sub>SC</sub>), respectively. The expression of adenovirally delivered HA-tagged WT-, CA-, and DN-CaMKII<sub>SC</sub> was determined by Western blotting with either an anti-HA or an anti-CaMKII antibody in cultured adult rat cardiomyocytes 24 h after infection (Fig. 1A). The autonomous activity of CaMKII, i.e. Ca<sup>2+</sup>-calmodulin-independent kinase activity, was assayed by 32P incorporation into a substrate peptide, was elevated by 5.8-fold in cardiomyocytes infected with Adv-CA-CaMKII<sub>SC</sub>, whereas it was suppressed by 70% in cardiomyocytes infected with Adv-DN-CaMKII<sub>SC</sub> (Fig. 1B). To further evaluate the functional consequence of manipulating CaMKII activity, we examined CaMKII-mediated PLB-Thr<sup>17</sup> phosphorylation. Consistent with the kinase activity profile, the expression of CA-CaMKII<sub>SC</sub> and DN-CaMKII<sub>SC</sub> caused a significant increase and decrease, respectively, in the phosphorylation of PLB-Thr<sup>17</sup> (32). These results indicate that we can specifically enhance or suppress CaMKII<sub>SC</sub> activity using adenoviral gene transfer techniques in cultured intact adult rat cardiac myocytes.

Increased CaMKII<sub>SC</sub> Activity Is Sufficient to Cause Heart Muscle Cell Apoptosis—We next explored the potential effect of CaMKII<sub>SC</sub> signaling on myocyte viability. Enforced expression of CA-CaMKII<sub>SC</sub> alone caused increased cardiomyocyte apoptosis, documented by increased TUNEL staining-positive cells (Fig. 2, A and B) and DNA fragmentation revealed by DNA laddering assay, whereas overexpression of WT-CaMKII<sub>SC</sub> did not induce apoptosis in these cells (Fig. 2, A and C), consistent with the kinase activity profile under the same experimental conditions (Fig. 1B). CA-CaMKII<sub>SC</sub>-mediated myocyte apoptosis occurred within 24 h after adenoviral gene transfer and increased in a time-dependent manner (Fig. 2B). Inhibition of CaMKII activity with the peptide inhibitor, AIP (5 μM), or co-expression of the DN-CaMKII<sub>SC</sub> mutant effectively suppressed CA-CaMKII<sub>SC</sub>-induced apoptosis (Fig. 2B). Furthermore, CA-CaMKII<sub>SC</sub> protein abundance and the kinase activity were closely correlated with the amount of Adv-CA-CaMKII<sub>SC</sub> virus delivered to the cultured cells (Fig. 3, A and B), with the severity of myocyte apoptosis correlating with the level of CaMKII activity (Fig. 3, C and D).

Involvement of Mitochondrial Death Machinery in Cardiomyocyte Apoptosis Induced by CaMKII<sub>SC</sub> Activation—To investigate whether the mitochondrial death pathway is involved in CaMKII<sub>SC</sub>-mediated heart muscle cell apoptosis, cytochrome c release into the cytosol was monitored in subcellular fractions by Western blotting. We found that cytochrome c was markedly increased in the cytosolic fraction but decreased in mitochondrial fraction in cardiomyocytes expressing CA-CaMKII<sub>SC</sub>, but not in those expressing DN-CaMKII<sub>SC</sub> or WT-CaMKII<sub>SC</sub> (Fig. 4, A and B), suggesting that enhanced CaMKII<sub>SC</sub> activity leads to cytochrome c release from mitochondria into the cytoplasm. The involvement of mitochondrial death signaling was further substantiated by the fact that overexpression of the anti-apoptotic Bcl-2 family member, Bcl-X<sub>L</sub>, suppressed CA-CaMKII<sub>SC</sub>-mediated cardiac myocyte apoptosis (Fig. 4, C and D).

Activation of Endogenous CaMKII by Various Stimuli That Trigger Myocyte Apoptosis—We next explored the possibility that other cell death-inducing stimuli, such as increased intracellular Ca<sup>2+</sup>- ATPase whose phosphorylation at PLB-Thr<sup>17</sup> serves as an intracellular marker of increased CaMKII activity, was augmented by 2–3-fold in myocytes...
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A. Control β-gal WT-CaMKII<sub>δC</sub> CA-CaMKII<sub>δC</sub> DN-CaMKII<sub>δC</sub>

B. CA-CaMKII<sub>δC</sub> + ADV CaMKII<sub>δC</sub> CA-CaMKII<sub>δC</sub> + AIP β-gal

C. CA-CaMKII<sub>δC</sub>

FIGURE 2. Inhibition of CaMKII activity by AIP or co-expression of DN-CaMKII<sub>δC</sub> protects cardiac myocytes against CA-CaMKII<sub>δC</sub>-induced apoptosis. A, representative micrographs show TUNEL staining in myocytes in the presence or absence of adenoviral gene transfer of CA-, DN-, WT-CaMKII<sub>δC</sub> or β-gal. In all experiments, the cells were infected with an adenovirus, as indicated, at m.o.i. 100. B, expression of CA-CaMKII<sub>δC</sub> triggered myocyte apoptosis in a time-dependent manner. Inhibition of CaMKII by co-expression of DN-CaMKII<sub>δC</sub> or the inhibitor, AIP (5 μM), significantly attenuates CA-CaMKII<sub>δC</sub>-mediated apoptosis. n = 3–4 independent experiments for each data point. * p < 0.05 versus all of the other groups. C, a representative example of DNA laddering in cultured cardiomyocytes infected with adenovirus expressing WT or mutant CaMKII<sub>δC</sub> for 72 h. Similar results were observed in four independent experiments.

FIGURE 3. Correlation between the activity of expressed CA-CaMKII<sub>δC</sub> and its evoked apoptosis in cultured cardiac myocytes. A, typical example of Western blot of HA-tagged CA-CaMKII<sub>δC</sub> assayed with anti-HA antibody. Similar results were observed in another three independent experiments. All of the experiments were performed after infection of cells, as indicated m.o.i. for 24 h, β-tetramer-dependent increase in CaMKII autonomous activity by infection of cells with Adv-CA-CaMKII<sub>δC</sub> for 24 h. * p < 0.01 versus control and β-gal. n = 3 for each group. C, time dependence of Adv-CA-CaMKII<sub>δC</sub>-mediated myocyte apoptosis after 72 h transfection.* p < 0.01 versus control and β-gal. n = 4–5. D, the elevation in the autonomous activity of CaMKII was closely correlated with the increase in the percentage of apoptotic cells with R<sup>2</sup> = 0.88.

DISCUSSION

In the present study, there are two novel findings. First, isoform-specific activation of CaMKII<sub>δC</sub> by stress caused by the administration of H<sub>2</sub>O<sub>2</sub> (12 μM), compared with untreated cells (Fig. 5), demonstrating that endogenous CaMKII activity was elevated in response to those treatments. The Ca<sup>2+</sup>-elevating agents markedly promoted myocyte apoptosis as assessed by increased TUNEL staining (Fig. 6A). On average, the percentage of apoptotic cells was increased ~3.5-fold. Similarly, intracellular acidosis or oxidative stress (H<sub>2</sub>O<sub>2</sub>, 12 μM) markedly augmented the percentage of TUNEL-positive cells (Fig. 6, B and C). Inhibition of CaMKII with KN-93 (2 μM) or AIP (5 μM) effectively protected cells not only from the Ca<sup>2+</sup>-elevating agents (Fig. 6A) but also against acidosis- and H<sub>2</sub>O<sub>2</sub>-mediated myocyte apoptosis (Figs. 6, B and C). In contrast, KN-92 (5 μM), an inactive KN-93 analogue, had no detectable protective effect (Fig. 6B). To define the relative contributions of CaMKII<sub>δC</sub> versus that of CaMKII<sub>δB</sub> to the observed cell death, we suppressed CaMKII<sub>δB</sub> to the observed cell death, we suppressed CaMKII<sub>δB</sub> to the observed cell death, we suppressed CaMKII<sub>δB</sub> to the observed cell death, we suppressed CaMKII<sub>δB</sub> to the observed cell death, we suppressed CaMKII<sub>δB</sub> to the observed cell death, we suppressed CaMKII<sub>δB</sub> to the observed cell death, we suppressed CaMKII<sub>δB</sub> to the observed cell death, we suppressed CaMKII<sub>δB</sub> to the observed cell death, we suppressed Ca MKII<sub>δC</sub> activity using adenoviral gene transfer of DN-CaMKII<sub>δC</sub>. Isoform-specific inhibition of CaMKII<sub>δC</sub> with the DN-CaMKII<sub>δC</sub> mutant, similar to isoform-irreversible inhibition of CaMKII with AIP, significantly reduced acidosis- or H<sub>2</sub>O<sub>2</sub>-mediated (20 μM) myocyte apoptosis (Figs. 7). Thus, increased CaMKII activity is associated with the administration of a number of different cell death-inducing stimuli. Expression of DN-CaMKII<sub>δC</sub> is able to inhibit the kinase activity and the associated cell death as well, indicating that activation of CaMKII<sub>δC</sub> is a common intermediate in the signaling pathways triggered by multiple stimuli that induce myocyte apoptotic death.

treated with Ca<sup>2+</sup>-elevating agents (1 μM ionomycin, 5 μM thapsigargin, or 60 mM extracellular K<sup>+</sup>), intracellular acidosis resulting from incubating cells with an acidic buffer with a [pH]<sub>i</sub> of 5.5 (with the equilibrated [pH], 6.6) (29), and oxidative expression of a constitutively active mutant is sufficient to trigger myocyte apoptosis. Second, inhibition of CaMKII effectively protects myocytes not only from adenoviral gene transfer of CA-CaMKII<sub>δ</sub> but also from those caused by
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increased intracellular Ca\textsuperscript{2+}, intracellular acidosis, and oxidative stress. These results support the conclusion that enhanced activation of CaMKII\textsubscript{AC} in cardiac myocytes is a common intermediate in the death signaling pathways initiated by diverse cell death-inducing stimuli.

Activation of Cardiac CaMKII\textsubscript{AC} Functions as a Common Intermediate Converging a Multitude of Cardiac Apoptotic Signaling Pathways—As is the case for most cells, upstream apoptotic signaling of cardiac myocytes can be classified into two general pathways: the death-ligand receptor-mediated pathway involving activation of caspase-8 and downstream executioner caspases and the mitochondrial or intrinsic pathway, the terminal steps of which involve the release of cytochrome \textit{c}, recruitment of apoptotic protease activating factor-1 (Apaf-1), and the activation of caspase-9 and downstream executioner caspases (33, 34). In contrast to the extrinsic pathway that transduces death signaling from a specialized set of death receptors, the intrinsic pathway integrates a broader spectrum of extracellular and intracellular stresses that activate signals converging on the mitochondria, leading to the release of a number of apoptogenic proteins that either directly or indirectly activate caspases. The present study has shown that activation of the cardiac cytosolic CaMKII isoform, CaMKII\textsubscript{AC}, is a common intermediate that integrates various apoptotic stimuli and relays the apoptotic signals to the mitochondrial death machinery, as evidenced by the robust increase in mitochondrial cytochrome \textit{c} release and the protective effect of Bcl-X\textsubscript{L}, an important anti-apoptotic member of Bcl-2 family (35).

Altogether, the present findings have identified a potentially crucial molecular intermediate involved in heart muscle cell loss and may shed new light on our understanding of the pathogenesis of heart failure and lead to a potentially important therapeutic approach for reducing myocyte loss, thus preventing or retarding the progression of heart failure caused by various etiologies.

Intracellular Distribution Dictates CaMKII Isoform-specific Functions—Recent work has demonstrated that stimulation of G protein-coupled receptors such as ET-1 leads to cardiac myocyte hypertrophy via a signaling pathway sequentially involving G\textsubscript{q}, phosphatidylinositol 1,4,5-trisphosphate-mediated nuclear Ca\textsuperscript{2+} release, activation of nuclear CaMKII (CaMKII\textsubscript{N}), HDAC5 phosphorylation, and activation of MEF2-dependent transcription (35). Remarkably, this Ca\textsuperscript{2+}-dependent, nuclear CaMKII-mediated hypertrophy signaling pathway cannot be activated by the global Ca\textsuperscript{2+} transients that cause myocyte contraction (36). Thus, it is reasonable to assume that the distinct intracellular localization of cardiac CaMKII\textsubscript{AC} and CaMKII\textsubscript{N} may enable myocytes to distinguish simultaneous local and global Ca\textsuperscript{2+} signals and exhibit different functional roles. This assumption is supported by studies in CaMKII transgenic mouse models. Specifically, overexpression of the cytoplasmic CaMKII\textsubscript{AC} isofrom leads to hyperphosphorylation of substrates involved in cardiac excitation-contraction coupling, resulting in SR Ca\textsuperscript{2+} leak and cardiomyopathy (37, 38), whereas overexpression of the nuclear CaMKII\textsubscript{N} isofrom leads to cardiac hypertrophic gene expression profile (21). These findings indicate that the intracellular distribution of the kinase dictates its accessibility and sensitivity to physiological and pathological stimuli.

Our previous studies have shown that overexpression of CaMKII\textsubscript{AC} but not CaMKII\textsubscript{N} enhances \beta\textsubscript{AR}-mediated cardiac myocyte apoptosis (25). The present study has further demonstrated that increased activation of CaMKII\textsubscript{AC} is sufficient to trigger cardiac myocyte apoptosis, whereas isofrom-specific inhibition of CaMKII\textsubscript{AC} is able to significantly protect cardiomyocytes against multiple death-inducing stimuli-mediated death. These findings provide an explanation for the observation of more severe heart failure and premature death phenotype in transgenic mice overexpressing cardiac CaMKII\textsubscript{AC} (37, 38, 39).
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A. 

\[ \text{P-PLB-Thr}^{17} \rightarrow \text{T-PLB} \]

B. 

Graph showing the phosphorylation of PLB-Thr\textsuperscript{17} in response to various death-inducing stimuli.}

A, a typical example of Western blot of phospholamban with an site-specific antibody reacting with phosphorylated PLB at Thr\textsuperscript{17}. The freshly isolated rat ventricle cardiomyocytes were treated with thapsigargin (5 \textmu M), ionomycin (1 \textmu M), high [K\textsuperscript{+}] (60 mM), H\textsubscript{2}O\textsubscript{2} (50 \textmu M) for 5 min, or a low pH (5.5) HEPES buffer for 30 min. The cell treated with isoproterenol (ISO, 0.1 \mu M) for 1 min as a positive control. B, average data of phosphorylation of phospholamban at Thr\textsuperscript{17} in rat cardiomyocytes.

38) compared with those overexpressing cardiac CaMKII\textsubscript{6B} (21). Future investigation is merited to better appreciate the distinct functional roles of these cardiac CaMKII isoforms and their regulation under a variety of physiological or pathological circumstances.

Potential Implications of CaMKII Deregulation in the Pathogenesis of Heart Failure—Multiple lines of evidence suggest that deregulation of CaMKII acts as an important pathogenic factor for heart failure, although this kinase plays a pivotal role in normal cardiac excitation-contraction coupling and pacemaker activity (10–16). First, previous studies have demonstrated that sustained \beta\textsubscript{1}AR stimulation, a characteristic of the failing heart, causes cardiomyocyte apoptosis via activation of CaMKII signaling, independently of the classic cAMP/cAMP-dependent protein kinase pathway (25). Second, CaMKII expression is increased in the failing hearts of humans and animals (5, 22–24). Third, transgenic overexpression of the cytosolic isoform CaMKII\textsubscript{6C} induces severe heart failure (37), which is associated with enhanced SR Ca\textsuperscript{2+} leak, reduced SR Ca\textsuperscript{2+} content and enhanced fractional SR Ca\textsuperscript{2+} release (37). Moreover, \beta\textsubscript{1}AR-induced fetal gene expression, a hallmark of cardiac hypertrophy, is mediated by a CaMKII-dependent mechanism in cultured rat neonatal cardiac myocytes (39). In contrast, inhibition of CaMKII activity prevents cardiac arrhythmias and suppresses after depolarizations, a crucial mechanism responsible for heart failure-associated arrhythmias (17, 18). Finally, CaMKII inhibition \textit{in vivo} improves cardiac functional performance and reduces catecholamine- and myocardial infarction-induced maladaptive cardiac remodeling (26). These studies have demonstrated that deregulation of CaMKII signaling is essentially involved in many aspects of cardiac hypertrophy and heart failure, marking CaMKII as a promising therapeutic target for the treatment of heart failure.

FIGURE 5. Increase in CaMKII-dependent phosphorylation of PLB at Thr\textsuperscript{17} in response to various death-inducing stimuli. A, a typical example of Western blot of phospholamban with an site-specific antibody reacting with phosphorylated PLB at Thr\textsuperscript{17}. The freshly isolated rat ventricle cardiomyocytes were treated with thapsigargin (5 \textmu M), ionomycin (1 \textmu M), high [K\textsuperscript{+}] (60 mM), H\textsubscript{2}O\textsubscript{2} (50 \textmu M) for 5 min, or a low pH (5.5) HEPES buffer for 30 min. The cell treated with isoproterenol (ISO, 0.1 \mu M) for 1 min as a positive control. B, average data of phosphorylation of phospholamban at Thr\textsuperscript{17} in rat cardiomyocytes. *, \( p < 0.01 \) versus control. \( n = 3–4 \).

FIGURE 6. Activation of endogenous CaMKII by various insults, including thapsigargin, ionomycin, high extracellular [K\textsuperscript{+}], intracellular acidosis, or H\textsubscript{2}O\textsubscript{2}, promotes cardiomyocyte apoptosis. A, average data of TUNEL staining in cultured adult rat cardiomyocytes subjected to thapsigargin (5 \textmu M), ionomycin (1 \textmu M), or high [K\textsuperscript{+}] (60 mM) for 24 h in the presence or absence of 30 min of pretreatment of cells with a CaMKII inhibitor, KN-93 (2 \textmu M), \( \beta \), average data of TUNEL staining in cells subjected to the normal (pH 7.4) or the acidic (pH 5.5) buffer. An inactive analogue of KN-93, KN-92 (2 \textmu M), was used as a negative control. C, average data of TUNEL staining in cells treated with H\textsubscript{2}O\textsubscript{2} (12 \textmu M) in the presence or absence of the pretreatment of KN-93. In all panels, inhibition of CaMKII with KN-93 significantly reduced all death-inducing stimuli mediated myocyte apoptosis. For A, *, \( p < 0.01 \) versus control and each corresponding pro-apoptotic treatment alone. For B and C, *, \( p < 0.01 \) as indicated. \( n = 3–4 \) independent experiments in cells from six hearts for each group.

FIGURE 7. Isoform-specific inhibition of CaMKII\textsubscript{6C} by adenoviral gene transfer of DN-CaMKII\textsubscript{6C} attenuates acidosis- and H\textsubscript{2}O\textsubscript{2}-induced cell death in cultured adult rat cardiomyocytes. 24 h after culture and adenoviral gene transfer of DN-CaMKII\textsubscript{6C} or \beta-gal (as a control adenovirus), the cells were treated with H\textsubscript{2}O\textsubscript{2} (20 \textmu M) for 24 h (A) or an acidic buffer, [pH] 5.5, for 8 h (B), and then TUNEL staining was performed. Expression of DN-CaMKII\textsubscript{6C} significantly reduced both H\textsubscript{2}O\textsubscript{2}- and intracellular acidosis-induced cell death. *, \( p < 0.01 \) as indicated. \( n = 4–5 \) independent experiments in cells from six to eight hearts for each group.
