

CaMKII δ Isoforms Differentially Affect Calcium Handling but Similarly Regulate HDAC/MEF2 Transcriptional Responses^{*[5]}

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The δ_B and δ_C splice variants of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII), which differ by the presence of a nuclear localization sequence, are both expressed in cardiomyocytes. We used transgenic (TG) mice and CaMKII expression in cardiomyocytes to test the hypothesis that the CaMKII δ_C isoform regulates cytosolic Ca²⁺ handling and the δ_B isoform, which localizes to the nucleus, regulates gene transcription. Phosphorylation of CaMKII sites on the ryanodine receptor (RyR) and on phospholamban (PLB) were increased in CaMKII δ_C TG. This was associated with markedly enhanced sarcoplasmic reticulum (SR) Ca²⁺ spark frequency and decreased SR Ca²⁺ content in cardiomyocytes. None of these parameters were altered in TG mice expressing the nuclear-targeted CaMKII δ_B . In contrast, cardiac expression of either CaMKII δ_B or δ_C induced transactivation of myocyte enhancer factor 2 (MEF2) gene expression and up-regulated hypertrophic marker genes. Studies using rat ventricular cardiomyocytes confirmed that CaMKII δ_B and δ_C both regulate MEF2-luciferase gene expression, increase histone deacetylase 4 (HDAC4) association with 14-3-3, and induce HDAC4 translocation from nucleus to cytoplasm, indicating that either isoform can stimulate HDAC4 phosphorylation. Finally, HDAC4 kinase activity was shown to be increased in cardiac homogenates from either CaMKII δ_B or δ_C TG mice. Thus CaMKII δ isoforms have similar effects on hypertrophic gene expression but disparate effects on Ca²⁺ handling, suggesting distinct roles for CaMKII δ isoform activation in the pathogenesis of cardiac hypertrophy *versus* heart failure.

Ca²⁺/calmodulin-dependent protein kinase II δ (CaMKII δ)⁴ is the predominant isoform of CaMKII in the heart. Splice variants differing in the presence of a nuclear localization sequence (NLS) show distinct subcellular targeting to either cytoplasmic or nuclear compartments (1–3). The CaMKII δ_B isoform contains an 11 amino acid NLS that is absent from δ_C . Thus CaMKII heteromers comprised predominantly of δ_B subunits localize to the nucleus while those with predominantly δ_C localize to the cytoplasm (1–3). We recently demonstrated that both δ_B and δ_C CaMKII are activated in response to pressure overload induced by thoracic aortic banding but that expression of these isoforms is differentially regulated (4). The possibility that there are discrete roles for these two isoforms in regulating Ca²⁺ homeostasis and gene transcription has not yet been explored.

CaMKII has long been implicated as a regulator of Ca²⁺ homeostasis and excitation-contraction (E-C) coupling in ventricular myocytes. This enzyme has been shown to phosphorylate proteins involved in sarcoplasmic reticulum (SR) Ca²⁺ handling including the cardiac ryanodine receptors (RyR2) and phospholamban (PLB) (4–10). Phosphorylation of the RyR2 appears to alter its channel open probability (9–11) while phosphorylation of PLB by CaMKII can regulate SR Ca²⁺ uptake (10, 12). Altered intracellular Ca²⁺ handling plays an important role in the pathogenesis of heart failure with changes in Ca²⁺ cycling preceding cardiac dysfunction. An emerging body of evidence has demonstrated that altered function of the RyR2, possibly due to increased phosphorylation by PKA, contributes to cardiac dysfunction in heart failure (13–16). Our previous studies demonstrated that expression of CaMKII δ_C in transgenic mice increased RyR2 phosphorylation and Ca²⁺ leak from the SR, and suggested that these were causal events in the development of heart failure and premature death (4). Increased CaMKII expression and activation in the RyR2 complex also contributes to the enhanced RyR2 phosphorylation and diastolic SR Ca²⁺ leak observed in an arrhythmogenic rab-

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⁴ The abbreviations used are: CaMKII, Ca²⁺/calmodulin-dependent protein kinase II; ANF, atrial natriuretic factor; BNP, brain natriuretic peptide; dn, dominant negative; GST, glutathione S-transferase; HDAC, histone deacetylase; MEF2, myocyte enhancer factor 2; β -MHC, β -myosin heavy chain; NLS, nuclear localization sequence; NRVMs, neonatal rat ventricular myocytes; PE, phenylephrine; PLB, phospholamban; RyR2, cardiac ryanodine receptor; SK-Actin, α -skeletal actin; SR, sarcoplasmic reticulum; TG, transgenic; WT, wild type; PBS, phosphate-buffered saline; HA, hemagglutinin.

bit model of heart failure (17). Whether the CaMKII δ_C isoform has more privileged access to and selectively phosphorylates these cytosolic substrates has not been addressed.

CaMKII has also been implicated in the transcriptional regulation associated with development of cardiac hypertrophy. We first reported that transient expression of δ_B CaMKII in neonatal rat ventricular myocytes induces atrial natriuretic factor (ANF) gene expression and results in enhanced transcriptional activation of an ANF-luciferase reporter gene (3). These data are consistent with the observation that the monomeric CaM kinases, CaMKI and CaMKIV, which like CaMKII δ_B can all enter the nucleus, also induce hypertrophic responses in cardiomyocytes *in vitro* (18). Several transgenic (TG) mouse models have now confirmed a role for CaMK in activation of the hypertrophic gene program and development of hypertrophy *in vivo*. Transgenic mice overexpressing calmodulin were found to develop severe cardiac hypertrophy (19) which was subsequently shown to be associated with an increase in the activity of CaMKII *in vivo* (20). Pronounced hypertrophy also develops in transgenic mice that overexpress CaMKIV (18) although CaMKIV is not a major CaMK isoform in the heart nor is it required for pressure overload induced hypertrophy (1, 21, 22). Our laboratory has shown that transgenic mice that overexpress the nuclear targeted cardiac CaMKII δ_B isoform develop hypertrophy, accompanied by cardiomyocyte enlargement and significant increases in hypertrophic gene expression (23). CaMKII δ_B is present and highly concentrated in cardiomyocyte nuclei of these TG mice (23), as it is when expressed in isolated cardiomyocytes (3).

The transcription factor myocyte enhancer factor 2 (MEF2) has been suggested to act as a common end point for hypertrophic signaling pathways in the myocardium (24, 25). Histone deacetylases (HDACs) have been shown to associate with and repress MEF2 activation (25–27) and signal-dependent dissociation of HDACs from MEF2 results in its activation (25, 27). MEF2 was demonstrated to be a downstream target for CaMK in CaMKIV TG (18) where MEF2 activation was suggested to occur through phosphorylation and dissociation of class II HDACs from MEF2. Whether there is a selective ability of the nuclear-targeted CaMKII δ_B isoform to control HDAC phosphorylation, MEF2 activation and hypertrophic gene expression *in vivo* and specifically in cardiomyocytes has not been explored.

The goal of the current study was to determine whether there are specific functions for CaMKII δ_B and CaMKII δ_C isoforms in the heart. We hypothesized that cytoplasmic CaMKII δ (composed of δ_C subunits) participates in phosphorylation and regulation of Ca²⁺ handling proteins, whereas nuclear CaMKII δ (composed of NLS-containing δ_B subunits) is selectively involved in regulation of HDAC phosphorylation and MEF2 activation associated with cardiomyocyte hypertrophy. Surprisingly, our findings demonstrate that whereas the δ_B and δ_C CaMKII isoforms have distinct effects on phosphorylation of Ca²⁺-handling proteins and Ca²⁺ regulation, the two isoforms similarly affect HDAC-mediated MEF2 and hypertrophic gene expression. These data suggest that the pattern of CaMKII isoform activation determines the propensity for development of

alterations in gene expression and Ca²⁺ handling that underly cardiac hypertrophy and heart failure.

EXPERIMENTAL PROCEDURES

Transgenic Mice—Transgenic mice expressing either the cytoplasmic CaMKII δ_C or the nuclear CaMKII δ_B in the heart were generated as described previously (4, 23). The creation of MEF2 indicator mice harboring a lacZ transgene controlled by three tandem copies of the MEF2 binding site (from the desmin enhancer) has been described elsewhere (28). CaMKII δ_B or δ_C TG mice were crossed with MEF2 indicator mice and offspring of WT and CaMKII δ_B or δ_C TG mice harboring MEF2-LacZ transgene were used for experiments. All mice used in the present study were at 4–5 weeks of age, unless otherwise noted. All procedures were performed in accordance with Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Western Blotting and Immunoprecipitation—Cardiac homogenates were prepared and Western blot analysis and immunoprecipitation carried out as described previously (4, 23). The antibodies used for immunoblotting and immunoprecipitation were as followings: mouse anti-RyR (Affinity Bioreagents), rabbit Ser2815 phospho-RyR2 antibody (a gift from Andy Marks laboratory, Columbia University, New York, NY), mouse anti-PLB (Upstate Biotechnology), rabbit anti-phospho-PLB (Thr¹⁷) (Cyclacel, Dundee, UK), rabbit anti-GFP, and mouse anti-14-3-3 β (Santa Cruz Biotechnology).

Isolation, Ca²⁺ Measurements and Immunocytochemical Staining of Adult Mouse Ventricular Myocytes—Mouse ventricular myocytes were isolated for Ca²⁺ spark frequency and SR Ca²⁺ content measurements as described previously (29). For immunocytochemical staining, isolated ventricular myocytes were plated on laminin-coated chamber slides and incubated for 1 h at room temperature. The cells were fixed using 100% ethanol (20 min at –20 °C), rinsed (three times) in phosphate-buffered saline (PBS) and then blocked for 1 h with 5% bovine serum albumin in PBS. Cells were incubated with primary antibody (HA antibody, Roche, dilution 1:60) in 1% bovine serum albumin in PBS containing 0.5% Triton X-100 overnight at 4 °C, then rinsed in PBS, and incubated with secondary antibody goat anti-mouse (Texas Red, Jackson Immuno Research) in 0.5% bovine serum albumin in PBS for 2 h at room temperature (dilution 1:200). Coverslips were mounted on slides by using Vectashield (Vector).

β -Galactosidase Staining and Activity Assays—Hearts from CaMKII δ_B or δ_C TG mice harboring MEF2-LacZ transgene or from mice expressing the MEF2-LacZ transgene but not CaMKII were collected and fixed in 4% paraformaldehyde buffered with PBS. Hearts were then immersed in Blueo-gal stain (3.1 mM ferricyanide, 3.1 mM ferrocyanide, 10 mM sodium phosphate, pH 7.2, 0.15 M NaCl, 1.0 mM MgCl₂, 1 mg/ml Blueo-gal in dimethylformamide) overnight at room temperature. β -Galactosidase activity assays were performed on ventricular extracts using a β -galactosidase assay kit from Stratagene under conditions of linearity with respect to time and protein concentration.

Cytoplasmic and Nuclear CaMKII in Calcium Handling and Transcriptional Regulation

Culture, Transfection, and Adenoviral Infection of Neonatal Rat Ventricular Myocytes—Neonatal rat ventricular myocytes (NRVMs) were prepared and transiently transfected or infected with adenoviruses as described previously (30, 31). Briefly, NRVMs were plated at a density of 4×10^5 cells per well of a 6-well plate, 4.5×10^6 cells per 10-cm dish or 5×10^5 cells per 3.5-cm dish and cultured overnight in serum-containing media. Cells were then washed and incubated in serum-containing medium for 2–4 h prior to transfections. Cells were cotransfected for 16–18 h with 3 $\times$ MEF2-luciferase reporter gene along with vector alone or vector encoding various CaMKII isoforms using a modified calcium phosphate technique (30). Myocytes were washed, the hypertrophic agonist phenylephrine (10 μ M) plus 2 μ M propranolol to block β -adrenergic response was added, and cells were incubated for an additional 48 h in serum-free medium. Luciferase activity in cell lysates was measured and normalized to total protein. For adenoviral infection, cells were washed after overnight culture and the medium was replaced with serum-free medium supplemented with insulin/transferrin/selenium (ITS). Cells were infected with AdCMV, CaMKII δ_B , CaMKII δ_C , and/or GFP-HDAC4 adenoviruses at 200–500 viral particles/cell for 16–18 h. The GFP-HDAC4 adenovirus was a gift from Martin Schneider's laboratory, University of Maryland, Baltimore, MD. Cells were subsequently washed and maintained in serum-free medium with supplements. After an additional 24–36 h, cells were harvested for immunoprecipitation studies or fixed for immunocytochemical staining with an anti-HA antibody (Roche Applied Sciences, 1:100 dilution) or an anti-GFP antibody (Santa Cruz Biotechnology, 1:100 dilution). Localization of the expressed HDAC4 was examined by immunostaining using confocal microscopy.

RNA Isolation and Real-time Reverse Transcription (RT)-PCR—Total RNA was prepared from mouse ventricular tissue using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System (Invitrogen) according to manufacturer's instructions. Real-time RT-PCR for the expression of hypertrophic marker genes was performed using TaqMan probes and primers from Applied Biosystems and the relative levels of expression were normalized to GAPDH.

HDAC4 Kinase Activity Assay—HDAC4 kinase activity assays were performed in ventricular homogenates as described previously (32) with minor modification. Briefly, a glutathione S-transferase (GST)-HDAC4 fusion protein (amino acids 419–670 of HDAC4) was used as a substrate. Another GST fusion protein with a R601F mutation in HDAC4 to prevent docking to CaMKII was used as control (32). The GST-HDAC proteins (500 ng) were conjugated to glutathione-agarose beads. GST-HDAC-bound beads were incubated with ventricular lysates (100 μ g protein) in lysis buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, and protease inhibitors) for 4 h at 4 °C. Beads were washed once with the same buffer. Beads were then resuspended in kinase reaction buffer (30 μ l; 25 mM HEPES pH 7.6, 10 mM MgCl₂, and 0.1 mM CaCl₂) containing 12.5 μ M ATP and 5 μ Ci [γ -³²P]ATP and reactions were allowed to proceed for 30 min at room temperature. Samples were boiled, and

phosphoproteins were resolved by SDS-PAGE, visualized by autoradiography, and quantified using a phosphorimager.

Statistical Analysis—All data are reported as mean $\pm$ S.E. Statistical significance of difference was determined using unpaired two-tailed Student's *t* test. *p* value <0.05 was considered statistically significant.

RESULTS

Phosphorylation of RyR2 and PLB at the CaMKII Sites in CaMKII TG Mice—To determine whether cytoplasmic and nuclear CaMKII differentially regulate the phosphorylation of Ca²⁺ regulatory proteins *in vivo* we examined phosphorylation of RyR2 and PLB in ventricular homogenates from CaMKII δ_B and δ_C TG mice. Our published studies used 3–4-month-old animals in which changes secondary to the development of hypertrophy or heart failure could obscure analysis of early signaling events. Accordingly in this study we examined young mice (4–5-week-old) and also directly compared two lines expressing similar amounts of either CaMKII δ_B or CaMKII δ_C . The phosphorylation of the RyR2, assessed using an antibody to the CaMKII phosphorylation site (Ser²⁸¹⁵ RyR2), was increased 2.2-fold in TG mice expressing the δ_C isoform of CaMKII (Fig. 1A). In contrast there was no increase in RyR2 phosphorylation in TG mice expressing the nuclear-targeted CaMKII δ_B (Fig. 1A). Phosphorylation of PLB at the CaMKII site (Thr¹⁷) was also significantly increased in CaMKII δ_C TG mice (Fig. 1B) but not in hearts from mice expressing nuclear-targeted CaMKII δ_B (Fig. 1B). This confirms our previous data suggesting that RyR2 and PLB are not *in vivo* targets for CaMKII δ_B but are targets for cytosolic CaMKII δ_C activity (4, 23).

SR Ca²⁺ Sparks and SR Ca²⁺ Content in CaMKII TG Cardiomyocytes—To determine whether the selective effects of the CaMKII isoforms on RyR2 and PLB phosphorylation resulted in functional differences in Ca²⁺ handling, adult cardiomyocytes were isolated from CaMKII δ_B and δ_C TG mice and Ca²⁺ spark frequency and SR Ca²⁺ content were assessed. The CaMKII δ_B transgene product was confirmed to be localized in the nucleus using anti-HA staining; that for CaMKII δ_C was shown to be concentrated in the cytoplasm of the isolated cardiomyocytes (Fig. 2A). Increased Ca²⁺ spark frequency (Fig. 2B) and decreased SR Ca²⁺ content (Fig. 2C) were observed in cardiomyocytes from the CaMKII δ_C TG mice, as we demonstrated earlier (4). The effects of nuclear-targeted CaMKII on these parameters were not previously reported. Here we show by direct comparison that spark frequency and SR Ca²⁺ changes are not observed in myocytes expressing CaMKII δ_B (Fig. 2, B and C), which localizes to the nucleus and fails to increase RyR2 phosphorylation. The cytoplasmic isoform of CaMKII δ_C thus appears to have a selective ability to directly alter Ca²⁺ handling via phosphorylation of Ca²⁺ regulatory proteins that are likewise confined to the cytosolic compartment.

In Vivo Measurement of MEF2 Activation by CaMKII—To determine whether the transcription factor MEF2 is an *in vivo* target for CaMKII δ , and explore whether its activation is specific for CaMKII signaling in the nucleus the CaMKII δ_B or δ_C TG mice were crossed with MEF2/ β -galactosidase indicator mice (28). MEF2 activation was detected by β -galactosidase

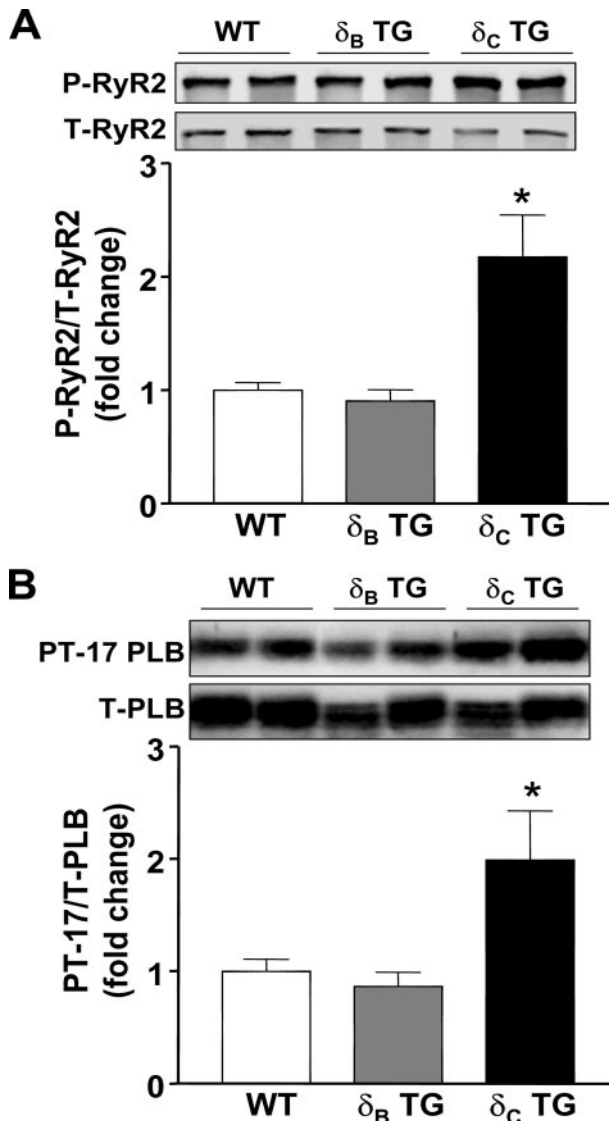


FIGURE 1. Increased RyR2 and PLB phosphorylation in CaMKII δ_C but not δ_B TG mice. A, phosphorylation of RyR2 at Ser²⁸¹⁵ was measured using Western blotting and normalized to total RyR2 in cardiac homogenates from CaMKII δ_B and δ_C TG mice. B, phosphorylation of PLB at Thr¹⁷ was measured using Western blotting and normalized to total PLB in cardiac homogenates from CaMKII δ_B and δ_C TG mice. *n* = 4 for each group. *, *p* < 0.05 versus WT.

staining of mouse hearts and by enzymatic assay of β -galactosidase activity in extracts from ventricles of WT, CaMKII δ_B and CaMKII δ_C TG mice harboring the MEF2-LacZ transgene. Young mice (4–5 weeks of age) which had not yet developed increased heart to body weight ratios or chamber enlargement were examined to ensure that observed changes in MEF2 activation were not secondary to these phenotypic changes. Hearts from WT mice expressing the MEF2/ β -galactosidase gene showed background β -galactosidase staining (Fig. 3A), reflecting basal MEF2 activity. β -Galactosidase staining of mouse hearts expressing CaMKII was markedly increased compared with WT (Fig. 3A). Unexpectedly, *in vivo* expression of either δ_B or δ_C isoforms of CaMKII increased the activation of MEF2 (Fig. 3A). To provide more quantitative assessment of β -galactosidase activity, ventricular extracts were prepared and β -galactosidase activity measured enzymatically as described under

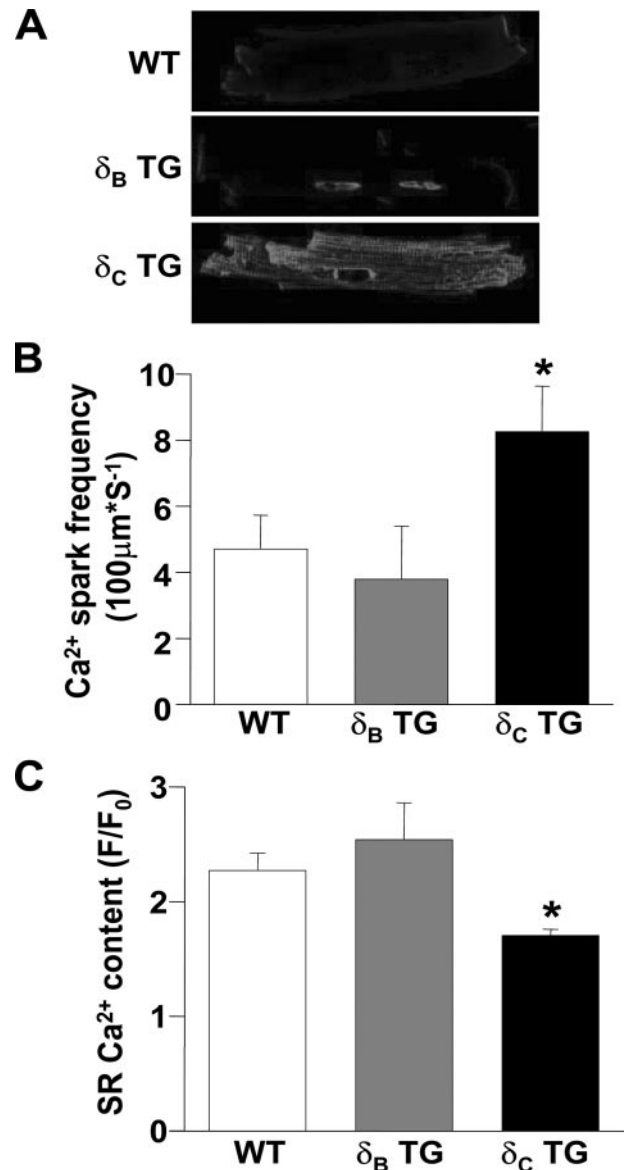


FIGURE 2. Selective ability of CaMKII δ_C to regulate Ca²⁺ handling. A, immunocytochemical staining of adult mouse ventricular myocytes from WT, CaMKII δ_B , and δ_C TG mice confirms the subcellular localization of the transgene, i.e. δ_B localized in the nucleus and δ_C in the cytoplasm. B, Ca²⁺ spark frequency was significantly increased in myocytes isolated from CaMKII δ_C TG mice but not CaMKII δ_B TG mice. C, SR Ca²⁺ content was significantly decreased in myocytes isolated from CaMKII δ_C TG mice but not CaMKII δ_B TG mice. *, *p* < 0.05 versus WT.

“Experimental Procedures.” Equivalent increases (~7-fold over WT) were seen in both the CaMKII δ_B and CaMKII δ_C expressing MEF2 reporter mice (Fig. 3B). The level of MEF2 protein expression was also assessed by immunoblotting and found not to differ in WT and TG mouse hearts (supplemental Fig. S1). Thus the observed effects of CaMKII on β -galactosidase activity indicate activation of MEF2-mediated transcription, not changes in MEF2 expression. These data demonstrate that increased expression of either δ_B or δ_C isoforms of CaMKII can drive MEF2-dependent genes *in vivo*.

In Vitro Measurement of MEF2 Activation by CaMKII—To more directly demonstrate that MEF2 can be activated by either CaMKII δ_B or CaMKII δ_C , MEF2-luciferase assays were per-

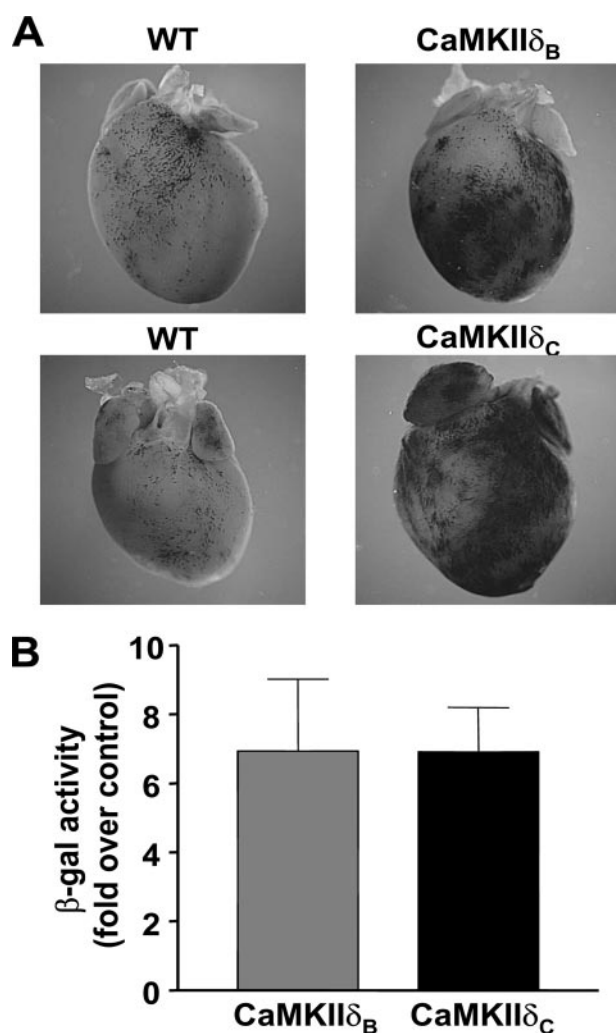


FIGURE 3. Either CaMKII δ_B or δ_C can activate MEF2 *in vivo*. *A*, CaMKII δ_B or δ_C TG mice were crossed with MEF2/ β -galactosidase indicator mice. β -Galactosidase staining of mouse hearts expressing either CaMKII δ_B or δ_C isoforms with the MEF2-LacZ transgene showed markedly increased staining compared with WT mice harboring the MEF2-LacZ transgene. *B*, β -galactosidase activity was measured in ventricular extracts from WT, CaMKII δ_B and δ_C TG mice harboring the MEF2-LacZ transgene. Equivalent increases (~ 7 -fold over WT) were seen in both CaMKII δ_B and CaMKII δ_C expressing MEF2 reporter mice ($n = 4$ for each group).

formed in neonatal rat ventricular myocytes (NRVMs). MEF2-luciferase activity, induced by the hypertrophic agonist phenylephrine (PE), was measured in the presence or absence of co-expressed WT or dominant negative (dn, K43A) forms of CaMKII δ_B or δ_C . The activation of MEF2 was enhanced by expression of either δ_B or δ_C CaMKII and inhibited by either dn CaMKII δ_B or δ_C (Fig. 4A). The involvement of HDAC in MEF2 activation by PE was also assessed by co-transfection of HDAC4. The ability of PE to stimulate MEF2 luciferase was inhibited by HDAC4 and the repression of MEF2 by HDAC4 was significantly attenuated by co-expression of either CaMKII δ_B or δ_C (Fig. 4B). These data indicate that CaMKII affects MEF2 activity through derepression of HDAC.

Induction of Hypertrophic Marker Genes in CaMKII TG Mice—We previously showed that a hypertrophic gene program and significant cardiac enlargement developed in both CaMKII δ_B and δ_C TG mice at 3 months of age (4, 23). The possibility that

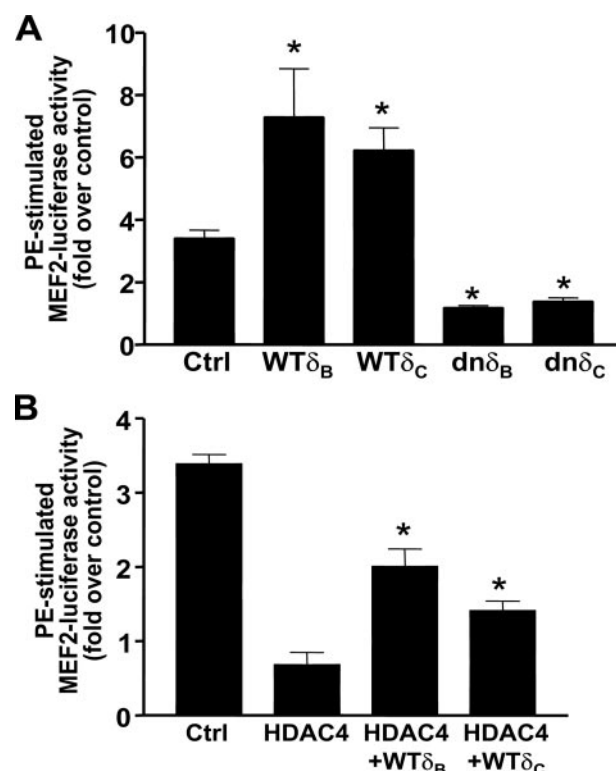


FIGURE 4. Either CaMKII δ_B or δ_C can activate MEF2 *in vitro*. *A*, neonatal rat ventricular myocytes (NRVMs) were transfected with an empty vector (Ctrl), WT CaMKII δ_B or δ_C , or dominant negative (dn) CaMKII δ_B or δ_C plus a 3 $\times$ MEF2-luciferase reporter gene. MEF2-luciferase activity was measured 48 h later in cells treated with the hypertrophic agonist phenylephrine (PE, 10 μ M). Values were normalized to total protein and the relative level of luciferase activity in the vector-transfected cells in the absence of PE was assigned a value of 1. *, $p < 0.05$ versus Ctrl. *B*, NRVMs were transfected with an empty vector as control (Ctrl), HDAC4 and HDAC4 with WT CaMKII δ_B or δ_C plus a 3 $\times$ MEF2-luciferase reporter gene. MEF2-luciferase activity was measured after 48 h of treatment with PE. Values were normalized to total protein and the relative level of luciferase activity in the vector-transfected cells in the absence of PE was assigned a value of 1. *, $p < 0.05$ versus HDAC4 alone.

the altered gene expression was a secondary response to changes in cardiac function was not ruled out in these studies. To determine whether hypertrophic marker genes were induced in both lines prior to development of other phenotypic changes, RNA from 4–5-week-old mice was analyzed by real-time RT-PCR. Hypertrophic marker genes including ANF, brain natriuretic peptide (BNP), β -myosin heavy chain (β -MHC), and α -skeletal actin (SK-Actin) were assessed. As shown in Fig. 5, increased expression of these hypertrophic marker genes was observed in both CaMKII δ_B and δ_C TG mice (except for SK-Actin). These findings support the idea that induction of hypertrophic genes is an early response to activation of MEF2 by CaMKII δ_B or δ_C .

HDAC4 Association with 14-3-3 and HDAC4 Translocation Regulated by CaMKII—CaMKII has recently been suggested to phosphorylate HDAC4 but not other class II HDACs (32). The phosphorylation of HDAC4 creates docking sites for the chaperone protein 14-3-3 and the phosphorylated HDAC4/14-3-3 complex is exported to the cytoplasm. To directly determine whether HDAC4 binding to 14-3-3 is a site of regulation by CaMKII δ and whether it is affected by both CaMKII δ_B and δ_C isoforms, cardiomyocytes were co-infected with CaMKII δ_B or δ_C and GFP-HDAC4 adenoviruses. Association of 14-3-3 with

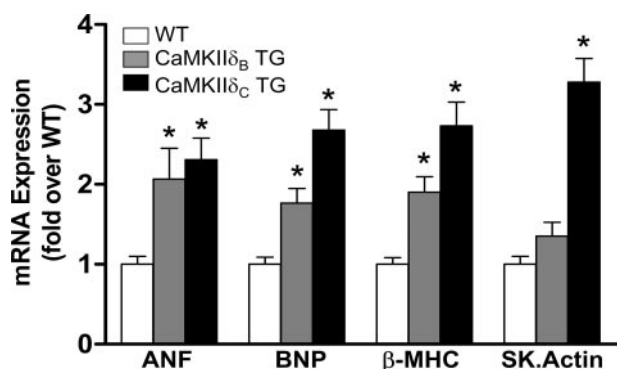


FIGURE 5. Induction of hypertrophic marker genes in young CaMKII δ_B and δ_C TG mouse hearts. Ventricular RNA was prepared from 4–5-week-old WT, CaMKII δ_B and δ_C TG mice ($n = 3–4$ for each group). Expression of hypertrophic marker genes for atrial natriuretic factor (ANF), brain natriuretic peptide (BNP), β -myosin heavy chain (β -MHC), and α -skeletal actin (SK-Actin) was assessed by real-time RT-PCR. GAPDH was used as the normalizing control. The relative level of mRNA in WT is assigned a value of 1. *, $p < 0.05$ versus WT.

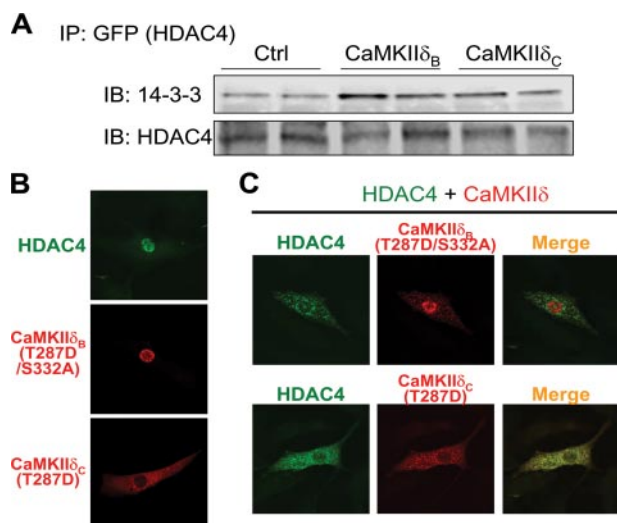


FIGURE 6. Both CaMKII δ_B and δ_C isoforms increase HDAC4 association with 14-3-3 and HDAC4 translocation from nucleus to cytoplasm. A, NRVMs were co-infected with AdCMV (Ctrl), CaMKII δ_B or δ_C and GFP-HDAC4 adenoviruses. After 36 h of infection, HDAC4 was immunoprecipitated from cell lysates and the HDAC-associated 14-3-3 protein analyzed by immunoblotting with a 14-3-3 antibody. A blot representative of three independent experiments is shown. Averaged data showed ~2.2-fold increase in 14-3-3 binding in CaMKII δ_B and ~1.7-fold in CaMKII δ_C -expressing cells. B, NRVMs were infected with HA-tagged constitutively active CaMKII δ_B (T287D/S332A), δ_C (T287D) or GFP-HDAC4 adenoviruses. Subcellular localization of HDAC4, CaMKII δ_B or δ_C in NRVMs was assessed by immunocytochemical staining with GFP or HA antibodies. HDAC4 was primarily localized in the nucleus. T287D/S332A CaMKII δ_B was also localized in the nucleus while T287D CaMKII δ_C was confined to the cytoplasm. C, NRVMs were infected with HA-tagged constitutively active CaMKII δ_B or δ_C along with GFP-HDAC4 adenoviruses. Subcellular localization of HDAC4, CaMKII δ_B or δ_C was assessed by immunocytochemical staining with GFP or HA antibodies. HDAC4 was translocated into the cytoplasm when co-expressed with either constitutively active CaMKII δ_B or δ_C isoform. Constitutively active CaMKII constructs were used in these studies because they gave more robust and readily quantifiable response than the WT constructs.

HDAC4 was assessed by co-immunoprecipitation experiments. As shown in Fig. 6A and replicate experiments, the amount of endogenous 14-3-3 associated with HDAC4 was increased ~2.2-fold and ~1.7-fold in CaMKII δ_B - and δ_C -expressing NRVMs respectively. Thus both CaMKII δ_B and δ_C isoforms can significantly enhance the association of HDAC4 with 14-3-3.

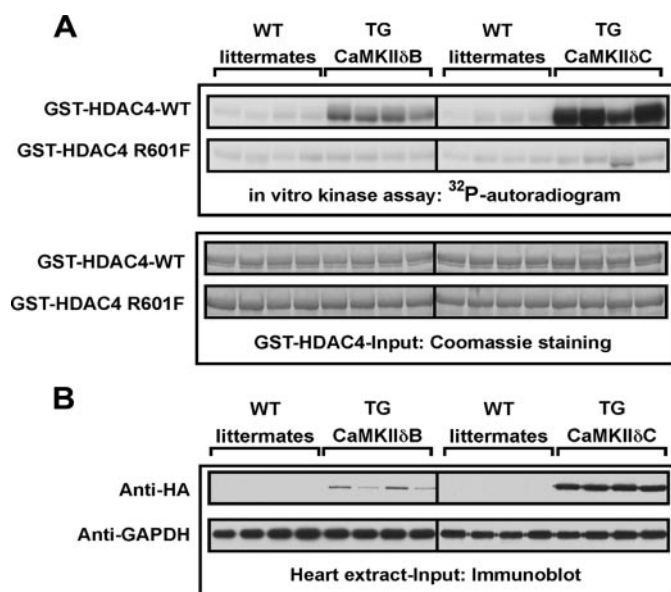


FIGURE 7. Increased HDAC4 kinase activity in both CaMKII δ_B and δ_C TG mouse hearts. A, HDAC4 kinase activity assays were performed in ventricular homogenates from CaMKII δ_B or δ_C TG mice and their WT controls using GST-HDAC4 peptide substrate or a GST-HDAC4 peptide substrate lacking the CaMKII docking site (R601F). HDAC4 kinase activity was significantly increased in homogenates from either CaMKII δ_B or δ_C TG mouse hearts compared with their WT controls. There was no increase in kinase activity assayed against HDAC4 lacking the CaMKII docking site (R601F). Coomassie staining was used to demonstrate equivalent GST-HDAC4 input. B, CaMKII transgene expression levels in the same samples from the two TG lines was assessed using an HA antibody.

The effects of CaMKII on HDAC4 translocation were then assessed. Constitutively active CaMKII δ_B (T287D/S332A) (32) and CaMKII δ_C (T287D) were used in these studies. As shown in Fig. 6B, GFP-HDAC4 is primarily localized in cardiomyocyte nuclei (~80% of cells showed prominent nuclear localization of HDAC4). The HA-tagged CaMKII δ_B was also localized in the nucleus, while the HA-tagged CaMKII δ_C was largely confined to the cytoplasm (Fig. 6B). Intriguingly HDAC4 was translocated into the cytoplasm when co-expressed with either isoform of constitutively active CaMKII δ (Fig. 6C). These data provide additional evidence that HDAC phosphorylation occurring in the cytoplasm (thus promoting its retention) can complement that occurring in the nucleus (leading to 14-3-3 binding and nuclear extrusion) and thereby contribute to the induction of MEF2-mediated gene expression.

HDAC4 Kinase Activity Assays in CaMKII TG Mice—CaMKII δ was recently shown to phosphorylate HDAC4 on Ser⁴⁶⁷ and Ser⁶³² (32). It has not been determined, however, whether CaMKII δ_B or δ_C are distinguishable in their ability to effect this phosphorylation or whether CaMKII expressed *in vivo* in cardiomyocytes has HDAC4 kinase activity. Accordingly, ventricular homogenates from CaMKII δ_B or δ_C TG mice and their WT controls were prepared and HDAC4 kinase activity assays were carried out using a GST-HDAC4 substrate containing the two CaMKII phosphorylation sites. As shown in Fig. 7A, HDAC4 phosphorylation was significantly increased in homogenates from either CaMKII δ_B or δ_C TG mouse hearts compared with their WT controls. There was no significant increase in HDAC kinase activity between either TG line and their WT littermate controls when HDAC5 was used as sub-

strate (supplemental Fig. S2). There was also no increase in phosphorylation of an HDAC4 substrate lacking the CaMKII docking site (R601F) (Fig. 7A), supporting the identity of the HDAC4 kinase measured in the ventricular homogenates as CaMKII. The higher CaMKII transgene expression levels in the CaMKII δ_C versus CaMKII δ_B mice used in these studies (Fig. 7B) likely explain their quantitatively greater effect on HDAC4 kinase activity, but strikingly and importantly, hearts from both lines showed robust HDAC4 kinase activity.

DISCUSSION

CaMK acts as a critical sensor for Ca²⁺ signals in the heart, potentially playing a central role in the development of cardiac hypertrophy and heart failure. CaMKII is a homo- or heteromultimer of 6–12 subunits encoded by α , β , γ , or δ genes (33, 34). The δ subunit of CaMKII predominates in the heart and there are distinct splice variants of CaMKII δ (δ_B and δ_C) characterized by the presence of a nuclear localization sequence in the variable domain (1, 35, 36). The NLS drives δ_B homomultimers or δ_B -enriched heteromultimers to the nucleus, whereas CaMKII δ_C subunit-rich multimeric enzyme would remain cytoplasmic. A hypothesis suggested by the differential localization of these CaMKII isoforms is that they play distinct roles in transducing signals that lead to hypertrophy and heart failure. In this study we addressed this question by examining the involvement of CaMKII δ in MEF2 gene expression, a specific transcriptional response implicated in hypertrophy, and in alterations of Ca²⁺ handling that could predispose to development of heart failure. Data obtained from a variety of experimental protocols and from both *in vitro* and *in vivo* experiments lead us to conclude that cytoplasmic and nuclear CaMKII δ isoforms share the capacity to induce HDAC-mediated MEF2 and hypertrophic gene expression, while selectively regulating phosphorylation of Ca²⁺-handling proteins and Ca²⁺ cycling.

Subcellular Sites of HDAC4 Phosphorylation and Transcriptional Activation by CaMKII—The premise with which we embarked on these studies was that HDAC4 would be phosphorylated and MEF2 activated within the nuclear compartment, the site of MEF2-dependent gene expression. Thus we expected these responses to be activated only by the nuclear-targeted CaMKII δ_B isoform. *In vitro* studies of MEF2-luciferase gene expression, of 14-3-3 binding to HDAC, and of accumulation of GFP-tagged HDAC4 in the cytoplasm all demonstrate, however, that these responses can be induced by forced expression of either δ_B or δ_C splice variants of CaMKII δ in cardiomyocytes. A recent publication from the Olson laboratory demonstrated that HDAC4 phosphorylation can be regulated either in the nucleus or in the cytosol of COS cells (32). This and another recent study (37), utilized activated forms of CaMKII that do not enter the nucleus and found them to stimulate MEF2 and regulate HDAC translocation. Our *in vivo* findings using TG mice provide further evidence for HDAC4 regulation by either CaMKII isoform. Specifically, data shown here demonstrate that a MEF2/ β -galactosidase reporter gene is transactivated, and that HDAC4 kinase enzymatic activity is increased, in hearts from TG mice expressing either CaMKII δ_B or CaMKII δ_C .

Class II HDACs act as transcriptional repressors of cardiac hypertrophy (38, 39), interacting with a variety of transcription factors in addition to MEF2, for example serum response factor (SRF). Overexpression of SRF can induce cardiac hypertrophy and dilation (40, 41), and CaM kinase has been shown to activate SRF by dissociating HDAC4 (42). Thus activation of CaMKII could regulate either MEF2- or SRF-dependent gene expression via phosphorylation and dissociation of HDACs. A range of hypertrophic genes are regulated by MEF2, SRF, and other transcription factors. We demonstrate here that there is comparable induction of hypertrophic marker genes, including ANF, BNP, and β -MHC, in hearts from CaMKII δ_B and CaMKII δ_C TG (Fig. 5). Thus our *in vitro* studies using MEF2-luciferase gene expression, 14-3-3 binding to HDAC, and HDAC4 translocation as well as *in vivo* studies demonstrating transactivation of MEF2, stimulation of HDAC4 kinase, and induction of hypertrophic gene expression all indicate that either CaMKII isoform can regulate gene expression.

It is possible that CaMKII δ_C might enter the nucleus as part of a heteromultimer with endogenous CaMKII δ_B and thus contribute to the induction of gene expression. Subcellular fractionation experiments revealed that a fraction of the overexpressed CaMKII δ_C is indeed associated with the nuclear fraction (supplemental Fig. S3). However, CaMKII δ_C is more highly distributed in cytosol, and less highly distributed in the nucleus than is CaMKII δ_B , yet it is the more efficacious isoform for inducing gene expression in the TG mouse lines (in Fig. 5). These data, together with the aforementioned evidence for cytosolic regulation of HDAC phosphorylation, argue strongly for complementary roles of the two CaMKII isoforms in controlling HDAC phosphorylation both from within and outside of the nuclear compartment.

Our previously published studies demonstrated that transient expression of the nuclear δ_B isoform of CaMKII induced ANF-luciferase reporter gene expression in neonatal rat ventricular myocytes whereas the cytoplasmic CaMKII δ_C did not (3). We recently repeated these experiments (data not shown) and found that ANF-luciferase reporter gene expression was induced in both CaMKII δ_B and δ_C transfected cardiomyocytes, consistent with the findings on MEF2 expression reported here. We suspect that the cDNA constructs used most recently (obtained from Schulman's laboratory, as were those used earlier) give higher levels of expression following transient transfection than those used previously. Whatever the explanation, expression of ANF-luciferase, like that of MEF2-luciferase and ANF mRNA, can be conferred by either CaMKII δ_B or CaMKII δ_C .

Mechanism of HDAC4 Regulation by CaMKII—Class II HDACs share a common structure and can be phosphorylated by CaMK and PKD. HDAC4 has recently been shown to have a specific CaMKII docking site that is not present on other HDACs (32). We show here that HDAC4 (but not HDAC5) kinase activity is significantly increased in CaMKII TG mouse hearts compared with WT controls. This profile is characteristic of CaMKII but not of CaMKI/IV or PKD which are general class II HDAC kinases that can phosphorylate and change subcellular localization of all class II HDACs (32).

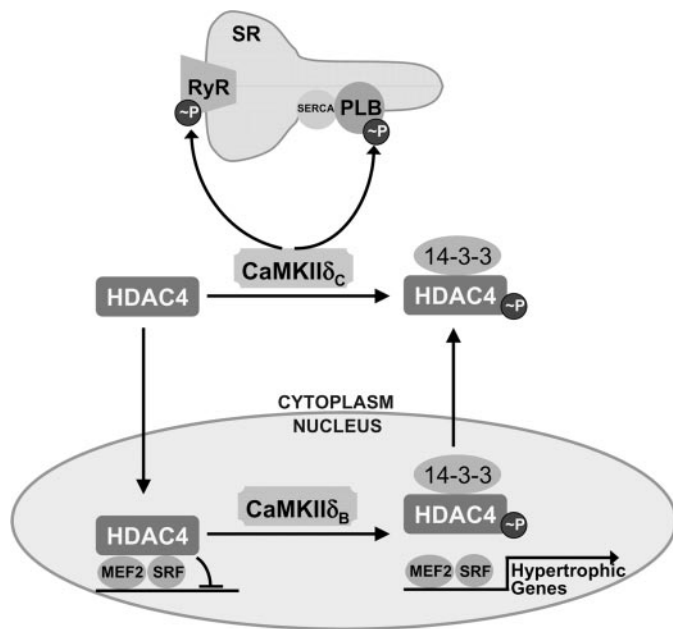


FIGURE 8. A schematic of cytoplasmic and nuclear CaMKII in regulating Ca^{2+} handling and HDAC/MEF2 transcription. Nuclear CaMKII (δ_B) is shown to phosphorylate HDAC4 in the nucleus, creating docking sites for the chaperone protein 14-3-3 and allowing the phosphorylated HDAC4/14-3-3 complex to be exported to the cytoplasm. MEF2 and other transcription factors are freed from HDAC repression and downstream genes are activated. Activation of cytoplasmic CaMKII (δ_C) is supposed to phosphorylate HDAC4 in the cytosol and prevent HDAC4 from going back to the nucleus. Thus cytoplasmic CaMKII could also derepress MEF2 and other transcription factors and thus lead to transactivation of their downstream genes. Activation of the cytoplasmic CaMKII (δ_C) can also phosphorylate Ca^{2+} regulatory proteins including RyR2 and PLB and mediate consequent changes in Ca^{2+} handling, events that are not controlled by nuclear CaMKII.

CaMKII-mediated phosphorylation of two serine sites on HDAC4 promotes its binding to 14-3-3 protein and accumulation in the cytoplasm. Interestingly, in our studies examining HDAC4 translocation we noted that when HDAC4 becomes cytoplasmic (in response to co-expression of CaMKII) it shows a punctuate distribution pattern (Fig. 6C). In addition, CaMKII δ_B , which is normally nuclear, appears to be partially translocated to the cytoplasm and co-localized with HDAC4. The cytosolic CaMKII δ_C also appears to show a punctuate pattern of localization when co-expressed with HDAC4 (Fig. 6C). Furthermore the CaMKII δ_C expressed in TG mouse hearts appears to be enriched in the perinuclear region (Fig. 2A) where we assume the blockade of HDAC4 import occurs. These findings suggest that CaMKII and phosphorylated HDAC4 form a signaling complex in the cytoplasm, and imply that whatever stabilizes this complex would have stimulatory effects on MEF2-mediated gene expression.

The model shown in Fig. 8 illustrates the proposed mechanism by which cytoplasmic CaMKII, phosphorylating HDAC4 in the cytosol (and presumably preventing it from re-entering the nucleus), can shift the equilibrium in much the same way as occurs when CaMKII phosphorylates HDAC4 in the nucleus and induces its nuclear export. While the detailed kinetics and regulation of the cytosolic and nuclear pathways may prove to be different, the ultimate effect of either mode of eliciting

HDAC4 accumulation in the cytosol would be to derepress MEF2-mediated gene expression.

Selective Effects of CaMKII Isoforms on Phosphorylation of Ca^{2+} -handling Proteins—In contrast to our findings with regard to HDAC4 phosphorylation and MEF2 gene expression, our data indicate that CaMKII δ_C is unique in its ability to phosphorylate RyR2 and PLB and to induce subsequent changes in SR Ca^{2+} stores and diastolic Ca^{2+} leak. We interpret the selective effect of CaMKII δ_C (versus δ_B) on phosphorylation of Ca^{2+} -handling proteins and Ca^{2+} regulation to reflect the cytoplasmic (versus nuclear) localization of the two CaMKII isoforms in cardiomyocytes. As noted previously, CaMKII δ protein expression is higher (17 versus 10-fold over WT), as is CaMKII activity (3- versus 1.5-fold over WT) in the CaMKII δ_C versus the CaMKII δ_B TG lines analyzed here (4, 23). Thus we cannot formally rule out that a higher level of CaMKII is necessary to phosphorylate PLB and RyR, a level exceeding that achieved in the CaMKII δ_B mice. However, in separate studies we established that the effects of CaMKII on phosphorylation and regulation of Ca^{2+} -handling proteins could be mimicked using acute adenoviral expression of CaMKII δ_C in adult rabbit cardiomyocytes (43) whereas comparable changes in Ca^{2+} handling were not observed when these cardiomyocytes were infected with CaMKII δ_B virus.⁵ These findings indicate that the selectivity of Ca^{2+} regulation by CaMKII δ_C in the transgenic mice is due neither to the level nor to the more chronic nature of CaMKII expression.

In our initial characterization of CaMKII δ_B TG mice we examined the phosphorylation of PLB (23). In agreement with the data presented here we found that PLB phosphorylation was not increased. Indeed, unexpectedly, PLB phosphorylation was actually decreased at both PKA and CaMKII sites (Ser¹⁶ and Thr¹⁷). Notably these studies were carried out in older mice (3–4 months of age), and in this report we concluded that the diminished phosphorylation could be accounted for by secondary increases in PP2A phosphatase activity associated with development of hypertrophy and ultimately heart failure (23). The present study compares young mice (4–5 weeks of age for both TG lines) studied before the onset of hypertrophy or heart failure. The lack of change in PLB phosphorylation in the CaMKII δ_B TG mice contrasts with the significantly increased PLB phosphorylation in CaMKII δ_C TG mice and suggests that PLB (like RyR) is accessible as a substrate only to cytoplasmic but not to nuclear-localized CaMKII.

Regulation of CaMKII Isoforms and Role in Cardiac Hypertrophy and Heart Failure—There are many possible mechanisms by which Ca^{2+} signals can be transmitted to the transcriptional machinery in the nucleus, a process that has been termed excitation-transcription (E-T) coupling. Ca^{2+} or Ca^{2+} /CaM can enter the nucleus and could, accordingly, directly activate their downstream nuclear targets (44). A more intriguing possibility derives from evidence of mechanisms for generating InsP₃ and eliciting Ca^{2+} signals within

⁵ M. Kohlhaas and L. S. Maier, unpublished data.

the nucleus (for reviews, see Refs. 45, 46) or in the nuclear envelope (10, 47, 48). A nuclear Ca^{2+} signaling cascade, including nuclear InsP_3 receptors and $\text{CaMKII}\delta$, could play a major role in coupling Ca^{2+} signals to transcriptional events in cardiomyocytes. Release of Ca^{2+} from InsP_3 receptor-regulated stores in the nuclear envelope would provide an efficient mechanism for activation of nuclear-targeted $\text{CaMKII}\delta_B$, independent of the changes in global Ca^{2+} that occur during the cardiac contractile cycle. It is also possible that a pool of cytosolic $\text{CaMKII}\delta_C$, localized in the vicinity of the nucleus, perhaps even complexed with InsP_3 receptors (47), is in a privileged position for selective activation by Ca^{2+} released through InsP_3 receptors and serves to support the induction of transcriptional responses.

Our previous work showed that δ_B and δ_C isoforms of CaMKII were both activated by pressure overload induced hypertrophy, but were differentially regulated at the expression level after longer times of pressure overload (4). The early increase in the activation of the two CaMKII isoforms is consistent with their both playing a causal role in the development of hypertrophy via transcriptional activation of MEF2-regulated genes. The initial activation of $\text{CaMKII}\delta_C$ may also serve a compensatory function by stimulating RyR2 and PLB phosphorylation and improving Ca^{2+} handling. More prolonged increases in cytoplasmic $\text{CaMKII}\delta_C$ expression, with concomitant increases in diastolic Ca^{2+} leak could, on the other hand, contribute to the downward spiral leading to heart failure by depleting Ca^{2+} stores and decreasing contractile function. MEF2- or SRF-regulated genes involved in development of dilated cardiomyopathy could also be up-regulated (40, 49). In this regard, the cytoplasmic $\text{CaMKII}\delta_C$ would appear to be an attractive and accessible therapeutic target to block development of heart failure.

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