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CaMKII δ mediates β -adrenergic effects on RyR2 phosphorylation and SR Ca²⁺ leak and the pathophysiological response to chronic β -adrenergic stimulation

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Abstract

Chronic activation of Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) has been implicated in the deleterious effects of β -adrenergic receptor (β -AR) signaling on the heart, in part by enhancing RyR2-mediated sarcoplasmic reticulum (SR) Ca²⁺ leak. We used CaMKII δ knockout (CaMKII δ -KO) mice, and knock-in mice with an inactivated CaMKII site S2814 on the ryanodine receptor type-2 (S2814A), to interrogate the involvement of these processes in β -AR signaling and cardiac remodeling. Langendorff-perfused hearts from CaMKII δ -KO mice showed inotropic and chronotropic responses to isoproterenol (ISO) that were similar to those of wild type (WT) mice, however in CaMKII δ -KO mice CaMKII phosphorylation of phospholamban and RyR2 was decreased and isolated myocytes from CaMKII δ -KO mice had reduced SR Ca²⁺ leak in response to isoproterenol (ISO). Chronic catecholamine stress with ISO induced comparable increases in relative heart weight and other measures of hypertrophy from day 9 through week 4 in WT and CaMKII δ -KO mice, but development of cardiac fibrosis was prevented in CaMKII δ -KO animals. A 4-week challenge with ISO resulted in reduced cardiac function and pulmonary congestion in WT, but not in CaMKII δ -KO or S2814A mice, implicating CaMKII δ -dependent phosphorylation of RyR2-S2814 in the cardiomyopathy, independent of hypertrophy, induced by prolonged β -AR stimulation.

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Disclosures

None declared.

Keywords

beta-adrenergic; CaMKII; fibrosis; hypertrophy; phosphorylation

Introduction

Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) is a multifunctional signaling molecule that has been implicated in pathological cardiac remodeling in response to stress [1]. CaMKII is known to be activated following Ca²⁺-dependent activation of calmodulin, through a variety of posttranslational modifications including autophosphorylation, oxidation, and O-linked glycosylation [2–4]. In response to activation of G-protein coupled receptors (GPCRs) by agonists such as endothelin or phenylephrine, CaMKII δ is phosphorylated at Thr287, possibly resulting from release of Ca²⁺ from InsP₃-sensitive stores [5]. Other GPCR agonists such as angiotensin, that are known to induce oxidative stress, lead to CaMKII activation via oxidation of Met281/282 [3]. There is growing evidence that β -AR stimulation can also activate CaMKII, although the signaling mechanisms through which this occurs remain controversial [6, 7]. Proposed mechanisms include activation of CaMKII through cAMP and PKA effects on Ca²⁺ channels [8], through Epac and PKC activation [9–11], through β -arrestin [12], or by a cAMP-independent mechanism which appears to involve nitric oxide [13, 14].

A relationship between β -adrenergic stimulation and CaMKII activity, especially in more chronic settings, is intriguing because both β -adrenergic and CaMKII pathways have been independently implicated in the development of heart failure [1, 6, 15–17]. Sustained β -AR stimulation resulting from increased sympathetic tone underlies the efficacy of β -AR blockers in heart failure treatment [17]. While CaMKII has not yet been therapeutically targeted in patients, increases in CaMKII expression and activation have been widely observed in animal and human models of heart failure [18–21]. Inhibition of CaMKII attenuates cardiac dysfunction and remodeling in mice subjected to sustained β -AR stimulation [16]. Furthermore, inhibition of CaMKII in human myocytes from patients with heart failure increases their contractile function [22]. Thus, CaMKII and its targets may represent major downstream signals for the pathological remodeling induced by chronic β -adrenergic stress.

The studies reported here utilize CaMKII δ knockout (CaMKII δ -KO) mice to specifically determine the role of CaMKII δ activity in the acute effects of β -AR stimulation on protein phosphorylation, sarcoplasmic reticulum (SR) Ca²⁺ handling, and inotropic/chronotropic responses. In addition we used CaMKII δ -KO mice to examine the effects of chronic β -AR stimulation on the development of hypertrophy and maladaptive remodeling. We demonstrate that deletion of the δ -isoform of CaMKII does not affect acute physiological β -adrenergic responses or the development of cardiac hypertrophy, but protects the heart against chronic isoproterenol (ISO) induced fibrosis and heart failure development. Moreover the maladaptive response to chronic β -AR stimulation, which is dissociated from its physiological and growth regulating effects, is shown to be dependent on CaMKII δ -mediated phosphorylation of RyR2-S2814.

Materials and Methods

Black Swiss mice in which the predominant cardiac isoform, CaMKII δ , was genetically deleted (conventional CaMKII δ -KO mice) were generated in our laboratory and characterized as described [15]. Cardiac-specific CaMKII δ -KO mice were generated by crossing heterozygous floxed CaMKII δ mice with MLC2v-Cre mice to ablate the CaMKII δ gene in ventricles as described [23]. Generation of C57/Bl6 RyR2-S2814A knock-in mice (S2814A) in which the CaMKII phosphorylation site was genetically inactivated has been described [24]. None of these mouse models showed gross baseline changes in ventricular structure or function compared to their respective wild type (WT) littermates. All mice used in the present study were male at 8–12 weeks of age, unless otherwise noted. Animal studies were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committees of University of California at San Diego and Davis, and Baylor College of Medicine.

Gene array and quantitative PCR

RNA extraction for microarray and Real time analysis were performed using the solid-phase RNeasy purification kit from Qiagen (Venlo, Netherlands). First strand cDNA synthesis for Real time PCR was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). A preoptimized Taqman Gene Expression Assays was used to quantify Col1a, Col3a, and glyceraldehyde-3-phosphate dehydrogenase mRNA as described by the manufacturer (Applied Biosystems). Gene expression was determined using Taqman® Universal PCR master mix, FAMTM-labeled Taqman® probe for collagen 1a and 3a and VICTM-labeled Taqman® probe for GAPDH (Applied Biosystems). Microarray analysis was done using the GeneChip Mouse Gene 1.0 ST Array and an GeneChip system comprising a Hybridization Oven 320, a GeneChip Fluidics Station 450, and a high-resolution GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA).

Isolation of adult mouse ventricular myocytes

Cardiac ventricular myocytes were isolated from the ventricles of 8–16-weeks-old WT or CaMKII δ -KO mice. Animals were anesthetized with pentobarbital, and hearts were removed, cannulated, and subjected to retrograde aortic perfusion at 37 °C, at a rate of 3 ml/min. Hearts were perfused for 4 min in Ca²⁺-free buffer, followed by 8–10 min of perfusion with 0.25 mg/ml collagenase (Blendzyme 1, Roche). Hearts were removed from the cannula and the ventricle was dissociated at room temperature by pipetting with increasingly smaller transfer pipettes. Collagenase was inactivated by resuspending the tissue in medium containing 10% bovine calf serum once the tissue was thoroughly digested. Calcium was gradually added back to a final concentration of 1 mmol/L and cells were plated on laminin-coated dishes in minimal essential medium/Hanks' balanced salt solution containing 5% serum. After 1 hr, cells were washed and serum-free medium was added back.

Line Scan Confocal Microscopy

Cells were isolated with the retrograde Langendorff perfusion technique as previously described [25]. Spontaneous Ca²⁺ sparks and SR Ca²⁺ load were assessed in freshly isolated cardiomyocytes loaded with 5 μ mol/L Fluo-4 AM (Molecular Probes). Before each

recording cells were paced at 1 Hz for 1 min to reach the steady state. Ca^{2+} sparks were assessed in quiescent intact cells. SR Ca^{2+} load was assessed as the amplitude of the Ca^{2+} transient induced by rapid application of 10 mmol/L caffeine after 1 min of steady state 1 Hz pacing. Recordings were performed using confocal microscopy (BioRad, Radiance 2100, x40 oil immersion objective) in line scan mode (3 ms/line). Excitation was via Ar laser (488 nm) and emission was collected at >505 nm. Image analyses were performed with Image J software and homemade routines in Interactive Data Language (IDL; ITT). All experiments were made at room temperature. All procedures were approved by the University of California Davis Institutional Animal Care and Use Committee.

CaMKII activity assay

CaMKII activity was measured in ventricular homogenate using Syntide-2, a synthetic CaMKII-specific substrate peptide. Hearts were isolated and ventricles homogenized in lysis buffer (50 mmol/L HEPES, 10% ethylene glycol, 2 mg/ml BSA, 5 mmol/L EDTA, pH 7.5), and assayed immediately without freezing. The assay buffer contained 50 mmol/L HEPES, 10 mmol/L magnesium acetate, 1 mg/ml BSA, 20 $\mu\text{mol/L}$ Syntide-2, 1 mmol/L DTT, 400 nmol/L [γ - ^{32}P]ATP, pH 7.5 and either 1 mmol/L EGTA (for autonomous activity) or 500 $\mu\text{mol/L}$ CaCl_2 , plus 1 $\mu\text{mol/L}$ calmodulin (for maximal activity). The reaction was carried out at 30 °C for 10 min and blotted onto Whatman P81 phosphocellulose paper. Percent activation was calculated as the ratio of autonomous to maximal activity for each sample.

Isolated Heart Studies

Mice were treated with heparin (500 U/kg i.p.) and anesthetized with pentobarbital (150 mg/kg i.p.). Animals were then killed, and hearts were quickly removed and placed in ice-cold Ca^{2+} -free Krebs-Henseleit buffer. Hearts were then placed on a Langendorff perfusion system (Radnoti LLC), and perfused at a constant pressure of 80 mmHg at 37 °C with a modified Krebs-Henseleit buffer solution containing (in mmol/L): 2.0 CaCl_2 , 130 NaCl, 5.4 KCl, 11 dextrose, 2 pyruvate, 0.5 MgCl_2 , 0.5 NaH_2PO_4 , and 25 NaHCO_3 and aerated with 95% oxygen and 5% carbon dioxide, pH 7.4. The left atrium was removed, and a water-filled balloon connected to a pressure transducer (MLT844, ADInstruments) was inserted into the left ventricle and inflated to 10 mmHg of LV end-diastolic pressure (LVEDP). Pre-agonist baseline data were recorded following a 20-min equilibration period. Subsequently, an ISO (1 $\mu\text{mol/L}$) infusion was initiated, and peak response was recorded. LV developed pressure (LVDP), the maximum rate of positive and negative change in LV pressure ($\pm\text{dP}/\text{dt}$), and heart rate were continuously recorded via a data acquisition system (Powerlab/8SP, ADInstruments). Hearts were flash frozen in liquid nitrogen and stored at -80 °C until processed. For assay of CaMKII activity hearts were homogenized and assayed immediately.

Histological analysis

Cardiac left ventricles were fixed for 24 hr in 4% paraformaldehyde dissolved in 0.1 M PBS (pH 7.4), embedded in paraffin, cut transversely into 5- μm sections, and stained with Masson's Trichrome. The area of fibrosis was quantified according to the method by Gaspard and Pasumarthi [26]. Briefly, images (10 $\times$ magnification) from sections were analyzed in Adobe Photoshop CS3 with the fuzziness of the color range (blue or red)

adjusted to the value 150. The percentage area of fibrosis was calculated using the equation: % fibrosis = blue pixels / (blue pixels + red pixels) × 100. To assess myocyte cross-sectional area, serial 5 µm sections were stained with TRITC-labeled wheat germ agglutinin (WGA; Sigma, St Louis, MO, USA) and nuclei with DAPI (Vector Laboratories, Burlingame, CA, USA). Myocyte cross-sectional area was quantitated using ImageJ software (version 1.48 g, NIH, Bethesda, MD, USA).

Immunoblotting

Western blot analysis from snap frozen ventricular tissue was performed according to protocols described previously [23]. The antibodies for immunoblotting were as follows: CaMKIIδ (D.M. Bers, UC Davis); PLN and α-actinin (Santa Cruz); GAPDH, total Akt, and Ser473 pAkt (Cell Signaling); phospho-PLN at CaMKII site (Thr17) and PKA site (Ser16) (Badrilla); RyR (Affinity Bioreagents); phospho-RyR2 at CaMKII site (Ser2814) and PKA site (Ser2808) (obtained from A.R. Marks, Columbia University, New York, New York, USA); ANP (US Biological); HDAC5 and Ser498 pHDAC5 (Assay Biotech).

Isoproterenol injection and osmotic pump delivery

Mice were treated with daily intraperitoneal injections of isoproterenol for 9 days (15 mg/kg/day) or 4 weeks (10 mg/kg/day) or isoproterenol was delivered for 2 or 4 weeks using implantable osmotic pumps for the continuous in vivo dosing of unrestrained mice (Alzet, Model 2004, Cupertino, CA). Pumps were loaded with isoproterenol to deliver 30 mg/kg/day (2 weeks) or 20 mg/kg/day (4 weeks), the animal was anesthetized, and a small incision was made for subcutaneous pump implantation on the back. The pump was inserted, delivery portal first in order to minimize interaction between the isoproterenol and the healing of the incision, and staples or surgical sutures were used for closure of the wound. In 4-week experiments, cardiac performance was evaluated every week for four weeks in the presence of isoproterenol then reevaluated during the fifth week without drug infusion.

Transthoracic echocardiography

CaMKIIδ-KO and WT control mice were anesthetized by isoflurane, 2% induction and 1–2% maintenance and echocardiography performed using an Agilent Technologies, Sonos 5500 with a 15 MHz linear probe. At least three independent M-mode measurements were obtained for each animal. Studies comparing S2814A and WT control mice were carried out in the Wehrens laboratory at Baylor College of Medicine using a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with high-frequency 30 MHz probe, as described [27]. All measurements were performed at a steady-state sedation level throughout the procedure (1.0% to 1.5% isoflurane mixed with 0.5 L/min 100% O₂) and in the presence of exogenous isoproterenol. Body temperatures were maintained between narrow ranges (37.0 ± 1.0 °C) to avoid confounding effects of hypothermia. Heart rate varied per mouse in response to isoproterenol.

Statistics

Results are presented as mean $\pm$ SEM, and comparison between groups was performed using ANOVA unless otherwise indicated. All statistical analyses were performed using Microsoft Excel software. A p-value < 0.05 was considered statistically significant.

Results

Characterization of CaMKII δ -KO mice

Adult male germline CaMKII δ -KO mice used in this study had no obvious basal phenotype when compared to WT mice, as reported previously [15]. Data obtained from Affymetrix arrays revealed that loss of CaMKII δ expression was not associated with compensatory mRNA upregulation of other CaMK isoforms (Fig. 1A). Deletion of two major splice variants of CaMKII δ , δ_B (which contains a nuclear localization sequence) and δ_C , was confirmed by Western blot (Fig. 1B). Lysates of ventricular tissue from CaMKII δ -KO mice were also assayed for CaMKII catalytic activity (at maximal activating conditions) and shown to have less than 20% of the total CaMKII activity seen in WT animals (Fig. 1C). This residual activity presumably reflects CaMKII isoforms other than CaMKII δ that are present at low levels in myocytes and in non-myocyte cell types in the heart.

Deletion of CaMKII δ reduces phosphorylation of Ca²⁺ handling proteins in adult cardiac myocytes

CaMKII δ phosphorylates a number of cardiac proteins involved in Ca²⁺ handling, including phospholamban (PLN) and the cardiac ryanodine receptor (RyR2). CaMKII and PKA share PLN and RyR2 as targets, but the phosphorylation sites are distinct and can be phosphorylated independently by the two protein kinases [28, 29]. To examine the effects of CaMKII δ gene deletion on the phosphorylation of PLN and RyR2 elicited by β -AR stimulation, we isolated cardiac myocytes from WT and CaMKII δ -KO hearts and treated them with 100 nmol/L isoproterenol (ISO) for 5 min. The myocytes were then harvested for Western blotting (Fig. 2). Representative Western blots (Fig. 2A, D) confirmed that the total levels of expression of PLN and RyR2 were unchanged in hearts from CaMKII δ -KO animals compared to WT controls. Myocytes from CaMKII δ -KO mice showed ~50–60% decreases in basal phosphorylation of PLN at the CaMKII site (Thr17) and RyR2 at the CaMKII site (Ser2814) compared to WT (CTL, Fig. 2B, E). There was no detectable decrease in basal phosphorylation at the PKA sites (pPLN Ser 16; pRyR Ser2808) in CaMKII δ -KO cells (CTL, Fig. 2C, F). Treatment of WT mouse myocytes with 100 nmol/L ISO lead to a robust increase in phosphorylation of PLN at the CaMKII site, which was significantly attenuated in CaMKII δ -KO cells (ISO, Fig. 2A, B). RyR2 phosphorylation at the CaMKII site was also increased by ISO in WT mouse myocytes and this response was significantly diminished in the CaMKII δ -KO cells (ISO, Fig. 2D, E). There was no effect of CaMKII δ deletion on phosphorylation of PLN at Ser16 or RyR at Ser2808 (Fig. 2C, F), consistent with these sites being regulated directly through β -adrenergic effects on PKA.

Deletion of CaMKII δ reduces diastolic SR Ca²⁺ leak

RyR2 phosphorylation at CaMKII sites has been linked to increases in diastolic SR Ca²⁺ leak through studies carried out in CaMKII transgenic and Epac2 knockout mice, as well as in phosphomimetic and non-phosphorylatable RyR2 mutant mice [19, 25, 30–32]. We used myocytes isolated from WT and CaMKII δ KO mice to directly examine the role of CaMKII in ISO mediated increases in Ca²⁺ sparks and diastolic Ca²⁺ leak (Fig. 3). ISO treatment led to a 2.5-fold increase in Ca²⁺ spark frequency (CaSpF) in myocytes from WT mice but failed to significantly increase Ca²⁺ sparks in CaMKII δ -KO cells (Fig. 3B). CaSpF and SR Ca²⁺ leak can be influenced by SR Ca²⁺ load (Fig. 3C and D), but CaSpF normalized for SR Ca²⁺ load was still increased 2.8 fold in WT and not increased significantly in CaMKII δ -KO (Fig. 3E). These data are consistent with the observed changes in RyR2-Ser2814 phosphorylation (Fig. 2) and its dominant role in regulating SR Ca²⁺ leak, and implicate CaMKII in ISO induced dysregulation of SR Ca²⁺ homeostasis.

Fight-or-flight response to acute β -AR stimulation is not altered in CaMKII δ -KO mice

To examine the role of CaMKII in β -adrenergic effects on the intact heart we isolated hearts from WT and CaMKII δ -KO mice and perfused them in the Langendorff mode as described [33]. We first used an enzymatic CaMKII activity assay to demonstrate that ISO treatment of isolated hearts activates CaMKII, as assessed by increases in the percent of autonomous (Ca²⁺ independent) activity. Indeed autonomous CaMKII activity was increased more than 2-fold (from 9 to 22% of total activity) at 10 min after addition of 1 μ mol/L ISO (Fig. 4A). Isolated heart function was studied at a fixed left ventricular end diastolic pressure of 10 mmHg. Baseline isovolumic cardiac performance was identical in isolated hearts from WT and CaMKII δ -KO mice, and ISO increased the rate and magnitude of pressure generation and the rate of pressure decay in hearts from both WT and CaMKII δ -KO animals. Representative baseline left ventricular dP/dt recordings and ISO responses are shown (Fig. 4B). Left ventricular developed pressure (LVDP) was increased by 43.8% in WT and to a comparable extent (44.8%) in CaMKII δ -KO (Fig. 4C). This agrees with the preserved β -AR-induced inotropic and lusitropic effects seen in CaMKII δ -KO myocytes [34]. The increase in heart rate (40.0% in WT, 45.8% in CaMKII δ -KO) was also not different (Fig. 4D).

Deletion of CaMKII δ has no effect on cardiac hypertrophy

The hypertrophic response of the mouse heart to β -AR stimulation with ISO is well described [35–37]. We used repeated daily injections or osmotic pump infusion of isoproterenol to determine if adult mice lacking CaMKII δ would be resistant to the hypertrophic effect of β -AR signaling. Wild-type and CaMKII δ -KO littermates were treated ISO for 9 days (Fig. 5A; 15 mg/kg/day i.p.), 2 weeks (Fig. 5B; 30 mg/kg/day s.c. infusion), or 4 weeks (Fig. 5C; 10 mg/kg/day i.p.). Wild-type mice exhibited significant increases in left ventricular mass in response to ISO. Daily injections of ISO for 9 days or 4 weeks led to 27 and 35% increases in heart weight to body weight ratio respectively, as compared to isovolumic vehicle (sterile saline) (Fig. 5A and C). Infusion of ISO resulted in a 28% increase in heart weight to body weight ratio as compared to control (Fig. 5B). Notably, CaMKII δ -KO hearts showed increases in heart/body weight that were equivalent to those of WT with all times and routes of ISO administration. Three other measures of cardiac

hypertrophy were also assessed. Phosphorylation of the class II histone deacetylase, HDAC-5, at serine 498 (which enables transactivation of hypertrophic genes) was significantly increased by ISO treatment in both CaMKII δ -KO mice and WT littermates (Fig. 5D). Atrial natriuretic peptide (ANP) showed significantly increased expression following ISO treatment in WT mice and this was not attenuated in the CaMKII δ -KO (Fig. 5E). The phosphorylation of Akt was determined in the same tissue samples and shown to not be increased by chronic ISO infusion in either WT or KO hearts (Suppl. Fig. 1) thus there is no evidence for development of a “physiological” hypertrophy in response to chronic ISO treatment. As a final measure, cardiac myocyte dimension was examined and found to be increased to the same extent in WT and CaMKII δ -KO hearts, confirming other evidence for unchanged pathological hypertrophy at the cellular level. The finding that the hypertrophic response to β -adrenergic stimulation is independent of CaMKII δ activation extends and is consistent with our previous observations that both pressure overload (TAC) and cardiac G α_q expression induce cardiac hypertrophy independent of CaMKII δ [15, 38].

CaMKII δ deletion inhibits fibrosis induced by chronic β -AR stimulation

The overall phenotypic changes induced by 2-week chronic infusion of ISO were relatively mild and did not result in heart failure development. However, infusion with ISO resulted in development of fibrosis in WT, which was significantly attenuated in hearts from CaMKII δ -KO mice (Fig. 6A). Increased synthesis and secretion of fibrillar collagen types I and III from cardiac fibroblasts plays a major role in the development of fibrosis. Accordingly, we assessed expression of collagen type I and type III mRNAs and found them to be markedly increased following chronic ISO treatment in hearts from WT but not CaMKII δ -KO (Fig. 6B).

To rule out the possibility that the marked difference in development of fibrosis resulted from loss of CaMKII signaling in fibroblasts, we also carried out studies using mice in which CaMKII δ was selectively deleted in cardiac myocytes. The cardiac-specific CaMKII δ -KO (CKO) mice were generated by crossing heterozygous floxed CaMKII δ mice with MLC 2v Cre mice. At baseline the CKO mice showed normal ventricular chamber dimension, wall thickness and cardiac function as assessed by echocardiography [23]. Using the cardiac specific CaMKII δ -KO mouse we confirmed that CaMKII δ deletion significantly impairs development of fibrosis in response to 4-week injection of ISO (Suppl. Fig. 2). Thus, the CaMKII δ -mediated β -AR signal that induces fibrosis appears to be cardiomyocyte autonomous. These findings suggest that CaMKII δ activation in cardiomyocytes contributes to the development of interstitial tissue abnormalities that accompany the progression of heart failure in the setting of chronic β -adrenergic stress.

CaMKII δ deletion protects from development of heart failure induced by sustained β -AR stimulation

While infusion of ISO for two weeks was not sufficient to induce heart failure, this was achieved in experiments in which mice received 4-week daily injections of ISO. Pulmonary congestion, an indicator of heart failure assessed by relative lung weight, was significantly increased by ISO treatment in WT mice and this response was significantly attenuated in CaMKII δ -KO mice (Fig. 7A). Furthermore echocardiographic analysis revealed severe LV

dysfunction, as indicated by reduced fractional shortening after four weeks of ISO treatment in WT, but not in CaMKII δ -KO mice (Fig. 7B). The CaMKII δ -KO mice subjected to ISO also showed a trend towards improved survival compared with WT (Fig. 7C). Fibrosis was significantly increased in hearts from WT mice injected with ISO for 4 weeks and this response was attenuated by CaMKII δ deletion (Fig. 7D).

Inactivation of the CaMKII phosphorylation site on RyR2 inhibits development of heart failure induced by chronic β -AR stimulation

To determine whether CaMKII-mediated phosphorylation of RyR2 contributes to the development of ISO-induced heart failure we examined the responses to chronic ISO in S2814A knock-in mice in which the major CaMKII phosphorylation site on RyR2 was genetically inactivated. These mice were previously shown to exhibit reduced cardiac remodeling and delayed progression of heart failure after TAC [30]. Studies were carried out using a protocol in which mice were subjected to continuous infusion of ISO (20 mg/kg/day) for a 4-week period. Echocardiographic measurements were carried out at various times following the ISO infusion protocol. Comparing 1 and 4 weeks of ISO treatment revealed that ISO treatment led to significant decreases in contractile function (Fig. 8A–D) and to development of cardiac dilation (Fig. 8E,F) in WT mice that were not significant in the S2814A mice.

Discussion

Role CaMKII in heart failure development

Our prior studies demonstrated that transgenic overexpression of the predominant cardiac isoform of CaMK, CaMKII δ , is sufficient to induce heart failure [19]. We subsequently reported that genetic deletion of CaMKII δ in mice did not inhibit pressure overload-induced hypertrophy but limited the transition from hypertrophy to heart failure [15]. Further studies established that CaMKII δ deletion also protected the heart against *in vivo* ischemia-reperfusion damage and conferred improved functional recovery [23]. In the present study we examined the role of CaMKII δ in responses to both acute and chronic β -AR stimulation. Acute β -AR activation effects on contractility and heart rate were unaltered in CaMKII δ -KO mice. This is consistent with previous data using isolated myocytes and demonstrating comparable inotropic and lusitropic responses to ISO in CaMKII δ -KO compared to WT cells [34]. Chronic ISO-induced stress also resulted in comparable cardiac hypertrophy in WT and CaMKII δ -KO mice. Recent studies from the Backs laboratory using a double knockout in which CaMKII γ , a more minor cardiac isoform, was genetically deleted along with CaMKII δ confirmed that hypertrophy in response to TAC or ISO were not CaMKII dependent [39]. Indeed they observed greater hypertrophy in the CaMKII double knockout, due to diminished inhibition of the calcineurin pathway.

Strikingly however, the development of cardiac fibrosis and progression to heart failure were inhibited in CaMKII δ -KO subject to chronic treatment with ISO, consistent with what we observed when these mice were subject to TAC [15]. More specifically, involvement of CaMKII in the progression of hypertrophy to failure following 6 week TAC [15] or 4 week ISO injection was evidenced by improved fractional shortening, less increase in lung/body

weight, and attenuated cardiac fibrosis. All of these data implicate CaMKII δ in β -AR induced cardiomyopathy. Pathological upregulation of fibrosis in cardiac tissue may predispose hearts to arrhythmias and cardiac arrest. This, along with acute arrhythmogenic effects of ISO, could explain the high mortality resulting from daily injection of ISO (only one third of the WT mice survived the 4 week ISO injection) and the somewhat diminished mortality seen in the CaMKII δ -KO mice (half survived the 4 week ISO injection). That significant mortality is still observed in the CaMKII δ -KO is likely due to the ability of ISO injection to still elicit β -AR and cyclic AMP-dependent inotropic and chronotropic responses. It has been reported that daily injections of ISO result in higher peak levels of ISO and greater hemodynamic stress than does ISO infusion [37]. The cardiomyopathic effects of ISO treatment also vary with the strain of mice being used [36, 40]. Differences in the genetic background of the RyR2814 versus CaMKII KO, as well as the use of 4 week ISO infusion (vs. injection) in studies using the RyR2814 mutant and WT mice, may explain why no mortality was seen in these mice.

RyR2 as a downstream target of CaMKII in ISO induced heart failure development

RyR2 is one of the major downstream targets of CaMKII δ in cardiac myocytes and is the main intracellular Ca²⁺ channel required for Ca²⁺ release from the SR. Enhanced CaMKII δ activity in failing hearts has been linked to diastolic SR Ca²⁺ leak in both human patients and in various animal models of heart failure [20, 30, 41]. CaMKII phosphorylates RyR2 at serine 2814 (S2814) and increases the open probability of the channel, irrespective of changes in SR filling, which may also be affected in failing hearts [42]. In our studies on TAC induced hypertrophy we demonstrated that phosphorylation of RyR2 at the CaMKII site and the associated Ca²⁺ leak were significantly increased at 6 week TAC and suggested that this could be a molecular mechanism contributing to the transition to heart failure. Further evidence that CaMKII-mediated RyR2 phosphorylation is critical for TAC induced heart failure development was provided by studies demonstrating that RyR2 S2814A mutant mice had significantly better cardiac contractility and exhibited less fibrosis than WT mice at 4 months after TAC [30].

Studies with intact rabbit ventricular myocytes demonstrated at the cellular level that β -adrenergic receptor agonists increase SR Ca²⁺ leak independent of PKA, whereas inhibitors of CaMKII (KN-93, or autocalmitide-2-related inhibitory peptide II) largely prevent the ISO-induced enhancement of SR Ca²⁺ leak [43]. This is consistent with the finding that CaMKII mediated phosphorylation of Ser2814 of RyR2 plays a predominant role in Ca²⁺ release from isolated SR vesicles after β -AR stimulation [44]. The current work extends these findings to the in vivo level demonstrating that the ability of chronic β -AR stimulation to elicit cardiac dilatation and reduced contractility is significantly diminished in S2814A knock-in mice. These data implicate CaMKII-mediated phosphorylation of RyR2 in the response to enhanced sympathetic tone and chronic β -AR stimulation, well known stress-induced regulators of cardiac dysfunction.

Role of CaMKII in abnormal calcium signaling

It may be challenging to accept, at face value, that increasing diastolic SR Ca²⁺ leak is sufficient to lead to heart failure development. It is likely, however, that this mechanism is

critical because it initiates and contributes to a vicious cycle in which CaMKII is a central player. Increased SR Ca^{2+} leak through CaMKII activation has been associated with an elevation of mitochondrial Ca^{2+} and subsequent development of heart failure and cardiomyocyte cell death [45, 46]. Elevated mitochondrial Ca^{2+} increases production of reactive oxygen species (ROS) which can also lead to posttranslational modifications in CaMKII that render it constitutively active [3]. Activated CaMKII can in turn affect mitochondrial Ca^{2+} and respiratory function through phosphorylation of a mitochondrial protein involved in mitochondrial calcium uniport, events which could further contribute to mitochondrial dysfunction and cardiomyocyte cell death [47]. CaMKII also has numerous other targets that could contribute to heart failure development including activation of cardiomyocyte NF- κ B signaling and inflammation and phosphorylation of ion channels and myofilament proteins [1, 23].

Our previous findings showed that CaMKII δ deletion inhibited apoptosis and associated cardiac fibrosis after 6 weeks of TAC [15] and CaMKII has been previously shown to mediate cardiomyocyte death in response to β -adrenergic stimulation [48–52]. We were, however, unable to document CaMKII-dependent changes in TUNEL staining in hearts from 2 week infused or 4 week injected mice. Thus while chronic ISO treatment could lead to CaMKII-mediated apoptosis and replacement fibrosis, a growing body of evidence suggests that viable cardiomyocytes, under stressed conditions, generate signals that promote interstitial fibrosis. For example, ATP release through pannexin 1 channels in cardiomyocytes contributes to the transformation of fibroblasts to profibrotic myofibroblasts following myocardial infarction [53]. Similarly, the fibrosis that occurs in pressure overload appears to require TGF β -receptor signaling in cardiomyocytes, as it is attenuated by cardiomyocyte-specific knockout of this receptor [54]. The histological analysis shown in the current study using the cardiac specific CaMKII δ -KO suggests that CaMKII localized in cardiomyocytes induces changes necessary for development of interstitial fibrosis during chronic β -AR stimulation (Suppl. Fig. 2). β -AR stimulation has been shown to increase TGF β_1 mRNA transcription and latent TGF β_1 release in cardiomyocytes [55, 56] thus CaMKII could stimulate fibrosis through effects on generation of this or other potent profibrotic factors.

Potential therapeutic implications of CaMKII inhibition in heart failure

β -AR blockade is an established therapeutic which inhibits excessive β -AR signaling and prevent worsening in left ventricular function following myocardial infarction [17]. Transgenic mice overexpressing a cardiac-specific peptide inhibitor of CaMKII, AC3-I, were shown to be significantly resistant to myocardial infarction-induced left ventricular dysfunction and dilatation [16]. The same study confirmed the beneficial effects using KN-93, a pharmacological inhibitor of CaMKII, and observed that the beneficial effect of CaMKII inhibition mimic salutary effects of β -AR blockers after myocardial infarction, and were independent of heart rate, because KN-93 did not reduce heart rate in their experiments [16]. The studies presented here use genetic deletion of CaMKII δ to provide direct evidence that CaMKII mediates the maladaptive effects of increased β -AR stimulation. Moreover results obtained using RyR2 S2814A mutant mice suggest a mechanism through which this occurs. Disabling CaMKII mediated RyR2 phosphorylation or inhibiting CaMKII would

appear to be promising approaches, used alone or in conjunction with β -AR blockers, to attenuate ventricular dysfunction in response to excessive β -AR in chronic heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We examined the role of CaMKII δ in cardiac β -adrenergic receptor signaling.
- Acute β -adrenergic responses to ISO are unchanged in CaMKII δ -KO mice.
- Chronic hypertrophic effects of ISO treatment are unchanged in CaMKII δ -KO mice.
- Chronic ISO-induced cardiac fibrosis and dysfunction are attenuated in CaMKII δ -KO.
- RyR2-S2814 phosphorylation and Ca²⁺ leak are involved in ISO induced cardiomyopathy.

Figure 1.

CaMK isoform expression and activity in CaMKIIδ-KO mice. A, mRNA transcript expression showing CaMK isoforms expressed in ventricular tissue from WT and CaMKIIδ-KO mice (n = 3 hearts each). The copy number was determined using the Affymetrix mouse gene array. B, Representative Western blot showing presence and absence of CaMKIIδ protein expression in left ventricular tissue isolated from WT and CaMKIIδ-KO mice, respectively. C, Total activity of CaMKII determined in lysates from WT and CaMKIIδ-KO hearts (n=6 each). The data are presented as the mean±SEM. *p<0.05 versus WT.

Figure 2.

Effect of CaMKII δ deletion on the phosphorylation of CaMKII targets in response to acute β -AR stimulation. A, Representative Western blot showing the phosphorylation of phospholamban (PLN) in adult mouse ventricular myocytes (AMVMs) harvested 5 min after stimulation with 100 nmol/L ISO or vehicle (CTL). B, Quantification of PLN phosphorylation at the CaMKII site Thr17. C, Quantification of PLN phosphorylation at the PKA site Ser16. D, Representative Western blots showing the phosphorylation of the ryanodine receptor (RyR2). E, Quantification of RyR2 phosphorylation at CaMKII site Ser2814. F, Quantification of RyR2 phosphorylation at PKA site Ser2808. Data are presented as mean $\pm$ SEM of the ratio of normalized phosphorylated protein over total protein (n=3–4 each). *p<0.05; **p<0.01; ***p<0.001 vs. CTL; #p<0.05 vs. WT.

Figure 3.

CaMKII δ is critical for ISO-induced SR Ca²⁺ leak. A, Representative Ca²⁺ spark recordings from WT cardiomyocytes $\pm$ isoproterenol (ISO; 100 nmol/L) and CaMKII δ -KO $\pm$ ISO (100 nmol/L) (represented as F/F₀ after background subtraction). Bright angled lines are Ca²⁺ waves and regions in red boxes are expanded to show sparks. B, Mean Ca²⁺ spark frequency (CaSpF) in intact cardiomyocytes isolated from WT (n=8 from 2 mice) and CaMKII δ -KO mice (n=7 from 2 mice), during control (CTL) conditions and after exposure to isoproterenol (ISO). C, Representative caffeine evoked Ca²⁺ transients (SR Ca²⁺ load) for WT (left) and

CaMKII δ -KO (right) in control condition (black) and in the presence of ISO (grey). D, Mean SR Ca²⁺ loads determined as in C for WT $\pm$ ISO (CTL: n=5 vs. ISO: n=7) and CaMKII δ -KO $\pm$ ISO (CTL: n=7 vs. ISO: n=10) from same animals as in B. E. CaSpF normalized to SR Ca²⁺ load in same cells as C-D. *p<0.01 vs. CTL; **p<0.001 vs. CTL (Student's t test).

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Figure 4.

Effects of acute ISO exposure (1 $\mu\text{mol/L}$) on CaMKII activity, contractility and spontaneous beating rate of isovolumic Langendorff perfused hearts. A, Autonomous CaMKII activity measured at steady state under control (CTL) conditions, and after 10 minutes of ISO perfusion. Activity of CaMKII was determined in fresh ventricular lysates from WT mouse hearts. B, Representative, simultaneously recorded dP/dt (mmHg/s) tracings showing changes of the rate of pressure development from steady-state baseline to maximum during ISO infusion. The arrows indicate start of ISO infusion. C, Group averages of left

ventricular developed pressure (LVDP). D, Group averages of spontaneous beating rate. Data are presented as mean $\pm$ SEM. n = 6 mice per group; *p<0.05 vs. CTL.

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Figure 5.

CaMKII δ deletion does not inhibit cardiac hypertrophy in response to chronic β -AR stimulation ($n = 5-9$ per group). A, Heart weight normalized to body weight (HW/BW) of WT and CaMKII δ -KO mice after 9 days (15 mg/kg/d i.p.), 2 weeks (30 mg/kg/d s.c. infusion), and 4 weeks (10 mg/kg/d i.p.) of continuous ISO treatment. D, Representative Western blot of HDAC5 phosphorylation at Ser498 in WT and CaMKII δ -KO hearts after 9 days of once-daily 15 mg/kg ISO injections. Quantification is shown to the right and is presented as the ratio of normalized phosphorylated HDAC5 over total HDAC5 ($n=3-4$ per group). E, Representative Western blot showing ANP protein expression in both WT and KO mice after 2 weeks of 30 mg/kg/d ISO infusion. Quantification is shown to the right ($n=3$ per group). F, Representative photographs of hearts from WT and KO mice stained with TRITC-labeled wheat germ agglutinin following 4 weeks of once-daily 10 mg/kg ISO

injection (n=3 per group). Quantification is shown to the right (300 cells counted per group). Data are presented as mean $\pm$ SEM; *p<0.05 vs. CTL; **p<0.01 vs. CTL.

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Figure 6.

CaMKII δ deletion inhibits cardiac fibrosis after 2-week continuous ISO infusion. A, Representative photographs of hearts stained with Masson's trichrome show collagen deposition, indicative of cardiac fibrosis (left). Quantification of cardiac fibrosis (right, n=4). B, Pro-collagen I (left) and III (right) mRNA expression in hearts from WT and CaMKII δ -KO mice (n=3 per group) after 2-week ISO infusion. Data are presented as mean $\pm$ SEM; *p<0.05 vs. CTL; #p<0.05 WT vs. KO.

Figure 7.

Effect of CaMKII δ deletion on the development of heart failure and cardiac fibrosis after 4-weeks of daily ISO injection. A, Lung weight normalized to body weight (LW/BW) of WT and CaMKII δ -KO mice (n=7–9 per group). B, Left ventricular fractional shortening (FS). C, Kaplan-Meier survival curves of WT and CaMKII δ -KO mice treated with ISO or vehicle. D, Representative photographs of hearts stained with Masson's trichrome show collagen deposition, indicative of cardiac fibrosis (left). Quantification of cardiac fibrosis is shown to the right (n=3 per group). *p<0.05 vs. CTL; **P<0.01 vs. CTL; #p<0.05 WT vs. KO.

Figure 8.

Echocardiographic parameters of wild-type and S2814A mice at one (1-wk) and four (4-wk) weeks of chronic ISO infusion. Data are presented as mean $\pm$ SEM. A, EF = ejection fraction; B, FS = left ventricular fractional shortening; C, ESD = end-systolic diameter; D, EDD = end-diastolic diameter; E, LVIDs = systolic left ventricular interior diameter; F, LVIDd = diastolic left ventricular interior diameter. * $p < 0.05$ vs. 1-week WT (one-tailed t test); # $p < 0.01$ WT vs. S2814A (two-tailed t test).