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SR-targeted CaMKII inhibition improves SR Ca²⁺ handling, but accelerates cardiac remodeling in mice overexpressing CaMKIIδ_C

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Abstract

Cardiac myocyte overexpression of CaMKIIδ_C leads to cardiac hypertrophy and heart failure (HF) possibly caused by altered myocyte Ca²⁺ handling. A central defect might be the marked CaMKII-induced increase in diastolic sarcoplasmic reticulum (SR) Ca²⁺ leak which decreases SR Ca²⁺ load and Ca²⁺ transient amplitude. We hypothesized that inhibition of CaMKII near the SR membrane would decrease the leak, improve Ca²⁺ handling and prevent the development of contractile dysfunction and HF. To test this hypothesis we crossbred CaMKIIδ_C overexpressing mice (CaMK) with mice expressing the CaMKII-inhibitor AIP targeted to the SR via a modified phospholamban (PLB)-transmembrane-domain (SR-AIP). There was a selective decrease in the amount of activated CaMKII in the microsomal (SR/membrane) fraction prepared from these double-transgenic mice (CaMK/SR-AIP) mice. In ventricular cardiomyocytes from CaMK/SR-AIP mice, SR Ca²⁺ leak, assessed both as diastolic Ca²⁺ shift into SR upon tetracaine in intact myocytes or integrated Ca²⁺ spark release in permeabilized myocytes, was significantly reduced. The reduced leak was accompanied by enhanced SR Ca²⁺ load and twitch amplitude in double-transgenic mice (vs. CaMK), without changes in SERCA expression or NCX function. However, despite the improved myocyte Ca²⁺ handling, cardiac hypertrophy and remodeling was accelerated in CaMK/SR-AIP and cardiac function worsened. We conclude that while inhibition of SR localized CaMKII in CaMK mice improves Ca²⁺ handling, it does not necessarily rescue the HF phenotype. This implies that a non-SR CaMKIIδ_C exerts SR-independent effects that contribute to hypertrophy and HF, and this CaMKII pathway may be exacerbated by the global enhancement of Ca transients.

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Disclosure Statement

No conflicts to disclose.

Keywords

transgenic mice; heart failure; Ca^{2+} /Calmodulin-dependent kinase; diastolic Ca^{2+} leak; Ca^{2+} sparks; excitation-contraction coupling

Introduction

CaMKII is crucially involved in excitation-contraction coupling (ECC) in the heart, and overexpression of the delta C isoform of CaMKII, the most prominent cardiac isoform [1], causes multiple defects in ECC and the rapid development of heart failure (HF) [2]. At three months of age mice overexpressing CaMKII δ_C show altered expression and phosphorylation levels of several Ca^{2+} handling proteins, severely reduced Ca^{2+} transient amplitude and sarcoplasmic reticulum (SR) Ca^{2+} content and greatly enhanced Ca^{2+} spark frequency (despite the lower SR Ca^{2+} content). The increase in Ca^{2+} sparks is indicative of an increased diastolic Ca^{2+} leak, which is known to occur in HF [3] and contributes to decreased SR Ca^{2+} load and hence cardiac pump function. The Ca^{2+} spark frequency in these mice could be acutely lowered by the CaMKII inhibitors KN93 and AIP, demonstrating that the increased spark frequency relied on continuously active CaMKII, most likely by direct CaMKII-dependent phosphorylation of the ryanodine receptor (RyR) [4]. Indeed, CaMKII δ knockout mice displayed a limitation in the transition to HF after transthoracic aortic banding, reduced CaMKII-dependent RyR phosphorylation and reduced SR Ca leak [5].

We hypothesized that the impairment in SR Ca^{2+} handling, and particularly the increased CaMKII-dependent RyR phosphorylation and leak are major contributors to the development of heart failure in the CaMKII δ_C mice. Accordingly we used targeted CaMKII inhibition at the SR membrane to test whether this would correct the defects in myocyte Ca^{2+} handling and thus prevent or limit HF in CaMKII δ_C mice. Engineered mice with SR-targeted CaMKII inhibition (SR-AIP) have been generated and previously studied by our group [6–8]. The inhibitor expressed in these mice is a fusion protein composed of four concatenated copies of the CaMKII inhibitor peptide AIP connected to a modified phospholamban (PLB) trans-SR membrane domain (mutated to prevent SERCA inhibition) which localizes the protein in the SR membrane [6–8]. These SR-AIP mice show only slightly impaired heart function (consistent with reduced PLB phosphorylation by CaMKII), but show no signs of HF unless stressed, e.g. by pregnancy [6,7]. We have previously reported that SR-AIP is effective in reducing CaMKII-dependent phosphorylation of two SR membrane proteins [6–8], and, most importantly, the expression of SR-AIP was associated with a reduced diastolic SR Ca^{2+} leak [8].

Here we show that in mice overexpressing CaMKII δ_C , co-expression of SR-AIP decreases diastolic SR Ca^{2+} leak and improves the impaired myocyte Ca^{2+} transients. However, while we expected to observe rescue of the disease phenotype, cardiac remodeling and functional deterioration are actually worse in crossbred mice. These data suggest that CaMKII signaling to non-SR CaMKII targets also contributes to dysfunction in CaMKII δ_C mice. It is also possible that the improved Ca^{2+} homeostasis directly enhances hypertrophic signaling and accelerates cardiac remodeling.

Methods

Transgenic mice

The generation and phenotype of the CaMKII δ_C overexpressing mice (CaMK) was previously described [2]. The transgenic line used for this study exhibits 3-fold increase in

phosphorylated CaMKII (indicative of the increased activity) and was shown to develop ventricular dysfunction within 2 months of age and profound dilated hypertrophy within 4 months of age.

The generation of SR-AIP mice was previously reported [6]. Briefly, SR-AIP mice express a synthetic gene containing four copies of the DNA sequence encoding the CaMKII inhibitor AIP (autocamtide-2-related inhibitory peptide) and a truncated PLB transmembrane domain (amino acids 23–52 and L31A/N34A) under control of the α MHC promoter (CaMK/SR-AIP). Mice of both genders and 3–4 months of age were used for all studies unless otherwise noted. Since WT vs. CaMK mice and vs. SR-AIP mice have been extensively compared previously [2,4,7–10], we focused on comparing CaMK versus CaMK/SR-AIP.

Myocyte isolation

Isolation of mouse ventricular myocytes was carried out essentially as previously described [11] and approved by the Loyola University Chicago animal welfare committee. Briefly, mice were anaesthetized with 5% Isoflurane in 100% O₂. Hearts were excised quickly and perfused according to Langendorff for ~5 min with nominally Ca²⁺-free MEM (M-0518, SIGMA, St. Louis, MO) supplemented with 10 mM HEPES, 10 mM Na-HEPES, 4.8 mM sodium bicarbonate, and 2 mM pyruvic acid. Additionally 20 units/l Insulin and 50,000 units/l Penicillin/Streptomycin were added and the pH adjusted to 7.35 with NaOH. Due to high spontaneous activity of the cells 10 mM BDM was included in the isolation media, but washed out before all experiments.

Western blot analysis

Quantitative immunoblot analysis was carried out following standard protocols. Protein (quantified via a modified Lowry assay; Pierce, Rockford, IL) was separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes (BioRad, Hercules, CA). Primary antibodies used were anti-RyR 5029 (RyR), anti-phospho-RyR Ser2814 (both generously provided by Dr. A. Marks, Columbia University, NY), anti-phospho-RyR Ser2808 and anti-phospho-PLB- Ser16 and Thr17 (Badrilla, Leeds, UK) and a custom anti-CaMKII δ antibody described previously [12]. Other antibodies used were anti-calsequestrin and anti-phospho-S286-CaMKII (both from Affinity BioReagents, Golden, CO), anti-protein phosphatase 1 (Chemicon, Billerica, MA), anti-protein phosphatase 2a (Upstate, Lake Placid, NY) and custom anti-SERCA2a and anti-PLB antibodies (both generously provided by Dr. M. Periasamy, The Ohio-State University, Columbus, OH). PLB was quantified as monomer in boiled samples. Protein expression was measured in myocyte lysates or heart homogenates, but phosphorylation levels were assessed in heart homogenates to avoid contribution of signal from dead/damaged cells to biochemical measures. Protein bands were visualized using the SuperSignal West Dura Kit (PIERCE, Rockford, IL). Signals were quantified using the UVP EpiChem³ imaging system and LabWorks 4.6 image acquisition and analysis software.

Tissue fractionation

Tissue was fractionated using differential centrifugation as described previously [13]. Briefly, flash frozen hearts were pulverized and homogenized using a Dounce glass tissue grinder. The homogenate was centrifuged for 10 min at 600 $\times$ g, the pellet washed three times and then resuspended in nuclear extract buffer (in mM: HEPES 20, 25% Glycerol, NaCl 420, MgCl₂ 1.5, EDTA 0.2). The resulting supernatant is the Nuclear Fraction. The supernatant from the first centrifugation was centrifuged at 5000 $\times$ g (to remove mitochondria) and the resulting supernatant then centrifuged at 100,000 $\times$ g. The supernatant is the Cytosolic Fraction and the pellet, after being washed 3 times and re-suspended in

RIPA buffer (in mM: NaCl 150, Tris 20, 1% Triton X 100, 0.1% SDS), is the SR/membrane or Microsomal Fraction.

[Ca²⁺]_i measurements

[Ca²⁺]_i was measured as previously described in field stimulated isolated cardiac myocytes at 23°C using the Ca²⁺ indicator dye fluo-4 [14]. SR-Ca²⁺ load was measured as the Δ[Ca²⁺]_i amplitude upon rapid exposure to 10 mM caffeine. SR Ca²⁺ leak was measured as the tetracaine-sensitive shift in [Ca²⁺]_i [15]. Briefly, SR Ca²⁺ leak is abruptly blocked by application of 1 mM tetracaine in Na⁺-free, Ca²⁺-free solution to block changes in intracellular Ca²⁺. The abrupt block of SR Ca²⁺ leak causes SR Ca²⁺ content to rise and cytosolic Ca²⁺ to decline, such that the [Ca²⁺]_i decline is directly indicative of SR Ca²⁺ leak. Cell shortening was measured continuously, using red light and a video edge-detection system (Crescent electronics, Sandy, UT, USA) as previously described [16].

Ca²⁺ Sparks in permeabilized myocytes

Myocytes were permeabilized in internal solution (in mM): K-aspartate 100, KCl 15, KH₂PO₄ 5, HEPES 10, MgATP 5, MgCl₂ 0.75, reduced glutathione 10, EGTA 0.4, CaCl₂ 0.12, creatine phosphokinase 5 U/mL, phosphocreatine 10, dextran (relative molecular mass: 40 000) 8%, K₄Fluo-4 0.035, pH 7.2 with KOH, containing saponin (50 μg/ml) essentially as described previously [11]. After 30 s the solution was exchanged with saponin-free internal solution and Ca²⁺ sparks were recorded as linescan images as previously described using a laser scanning confocal microscope (Radiance 2000 MP, Bio-Rad, UK). SR Ca²⁺ load was evaluated by rapid application of caffeine. Spark recordings were analyzed with SparkMaster [17] and the properties of 20% of the sparks with the highest amplitude averaged (to eliminate out-of-focus events, [18]).

In vivo 2D guided M-mode echocardiography

Echocardiography was performed essentially as described elsewhere [19,20]. Mice were anesthetized with isoflurane and measurements were performed using a Toshiba Aplio 80 Diagnostic ultrasound System (Model SSA-77A, PST65AT and PLT-1202S transducers) according to American Society of Echocardiography guidelines. Left ventricle diastolic and systolic wall thickness, left ventricle end-diastolic diameter (LVEDD) and left ventricle end-systolic diameter (LVESD) were measured. The percentage of LV fractional shortening (%FS) was calculated as [(LVEDD-LVESD)/LVEDD]×100.

Histological/morphometric analysis

After short in situ perfusion with 10% buffered formalin 1-month old mouse hearts were post-fixed for 48 hours in 10% formalin, paraffin-embedded and cut into 4 μm sections. One set was stained with periodic acid-Schiff (PAS) and hematoxylin to outline cell borders and define nuclei respectively and another set was stained with Masson's trichrome to examine fibrosis. Five images per heart were recorded using a Leica DM IRB inverted microscope with a Nikon DXM1200C camera using identical settings for all images. Analysis was performed with ImageJ 1.43 (NIH), in part by using automated image thresholding and particle analysis functions.

Statistical analysis

Results are expressed as means ± S.E. Significance was estimated by one way ANOVA followed by Bonferroni post-hoc tests, one-way repeated measures ANOVA and/or Student's t-test for paired observations as appropriate. P ≤ 0.05 was considered significant.

Results

Transgene expression

The mutated PLB transmembrane domain used here to target AIP to the SR membrane (together SR-AIP) is not able to inhibit SERCA pump by design, but these mutations still allow SR-AIP to oligomerize with endogenous PLB. SR-AIP has a higher molecular weight than PLB monomer and when SR-AIP is incorporated into pentamers with PLB, the mobility of the pentamer is slowed in SDS-PAGE (Fig 1A). The presence of PLB/SR-AIP oligomers as higher molecular weight bands containing PLB confirms SR-AIP expression in the SR membrane (Fig. 1A). Three to four distinct bands were observed (presumably containing from 0 to 3 molecules of SR-AIP per pentamer). CaMKII δ C overexpression was not affected by simultaneous expression of SR-AIP in the double transgenic mice (Fig. 1B–C). Subcellular fractionation revealed that the distribution of total CaMKII δ C between nuclei, microsomal and cytosol fractions was not altered by SR-AIP expression (data not shown). Consistent with an inhibitory effect of SR-AIP on CaMKII at the SR membrane, CaMKII Thr287 phosphorylation in the membrane fraction was reduced to 67.7 ± 2.2 % of the level in CaMK mice (Fig. 1D). This value likely underestimates the effectiveness of inhibition of CaMKII autophosphorylation at the SR membrane, because this microsomal preparation also contains fragments from other membranes (e.g. sarcolemma). Despite this, total cellular CaMKII autophosphorylation was not decreased, and if anything higher (although not significantly so, Fig 1E). To explain this quantitative discrepancy, CaMKII autophosphorylation would have to be elevated in some non-SR compartment or subcompartment, although this was not detectable in overall nuclear or cytosolic fractions (not shown).

Expression of Ca²⁺ handling proteins

We previously found that development of HF in CaMK mice was associated with altered expression levels of major Ca²⁺ handling proteins, with decreases at 3 months of age in SERCA (–30%), PLB (–20%) and RyR (–58%) expression levels [4]. We tested whether co-expression of SR-AIP in CaMK mice restores expression levels of these proteins toward WT levels. SERCA (96 ± 4 % of CaMK in CaMK/SR-AIP, n=4) was similar between groups, thus 30% lower than in WT in both groups, and also calsequestrin expression (97 ± 1 % of CaMK in CaMK/SR-AIP, n=4) was not altered. In contrast, PLB expression (Fig. 2A) was increased in double-transgenic mice and therefore near WT levels [4]. On the other hand, RyR expression was further decreased in double transgenic mice to 77 ± 4 % vs CaMK (this means dramatically decreased to 32 ± 2 % of WT; Fig. 2C). We also measured PLB and RyR phosphorylation using phosphorylation-site specific antibodies in whole heart homogenates (Fig. 2A–E). As expected, CaMKII mediated phosphorylation at T17 on PLB was decreased by SR-AIP expression (Fig. 2B) as described previously in the absence of CaMKII δ C overexpression [6].

The total amount of S2814 phosphorylated RyR was reduced in CaMK/SR-AIP mice (Fig 2D) and consequently may explain reduced SR Ca leak. However, when normalized to RyR expression the relative RyR-PS2814 was unaltered by SR-AIP (Fig. 2E), which is not what was observed when SR-AIP alone was expressed (Fig. 2D, [8]). SR-AIP co-expression also affected phosphorylation sites phosphorylated by PKA, because PLB-PS16 was increased to 281 ± 24 % (n=8), and total and relative phosphorylation at PKA site RyR-PS2808 was decreased (to 70 ± 4 % and 83 ± 4 % respectively; Fig. 2 D+E). These effects did not result from secondary changes in the expression levels of major phosphatases in the heart since global expression of the catalytic subunits of protein phosphatases 1 and 2a was comparable in both groups (PP1: 100 ± 6 vs 111 ± 6 (n=8) and PP2a: 100 ± 9 vs 113 ± 11 in CaMK vs SR-AIP/CaMK respectively).

Ryanodine receptor function in permeabilized myocytes

RyR function was investigated by measuring Ca^{2+} sparks in saponin-permeabilized cardiomyocytes. This technique allows us to study elementary Ca^{2+} release events while controlling diastolic $[\text{Ca}^{2+}]$ and eliminating effects of the L-type Ca^{2+} channel on RyR. We analyzed only 20% of the brightest sparks to eliminate out-of-focus release events [18], but our conclusion is unaltered when all ~10,000 detectable sparks were included in the analysis (not shown). Frequency and amplitude of Ca^{2+} sparks were similar in both groups (table 1) and SR Ca^{2+} load measured by rapid application of caffeine was not significantly altered (i.e. increased SR Ca load increases Ca spark frequency). However, we found that co-expression of SR-AIP decreased the spark size (width and duration), resulting in decreased spark signal mass (amplitude $\times$ 1.206 $\times$ FWHM³ [21]) in CaMK/SR-AIP vs. CaMK myocytes. Accordingly, the spark mediated SR Ca^{2+} leak (signal mass $\times$ spark frequency) was decreased by approximately 19% (Table 1). Note that in CaMKII δ_{C} vs. WT the Ca spark frequency was dramatically increased in intact myocytes (4-fold increase in spark-mediated leak [6]). While we cannot directly compare those intact cell results with those here in permeabilized myocytes, the amount of Ca^{2+} leak via sparks in CaMK/SR-AIP probably still exceeds the WT level.

Decreased diastolic SR Ca^{2+} leak

The diastolic SR Ca^{2+} leak was also quantified in intact myocytes as described previously [15]. This procedure measures the decrease of $[\text{Ca}^{2+}]_i$ in the presence of the RyR blocker tetracaine while the overall cellular Ca^{2+} content is maintained (blocked Ca^{2+} influx and efflux, see Fig. 3A). In CaMK/SR-AIP myocytes Ca^{2+} leak was decreased by about 50% (from 19.0 ± 1.8 nM (n=17) in CaMK to 8.7 ± 1.1 (n=20) in CaMK/SR-AIP) while at the same time the SR Ca^{2+} load was increased from 205 ± 5 (n=17) to 219 ± 5 $\mu\text{mol/L}$ cytosol (n=20) (Fig. 3B). We have shown that the leak is strongly dependent on the SR Ca^{2+} load, and increases steeply at higher Ca^{2+} loads [15]. Thus, even though the SR Ca^{2+} load was increased, the leak was reduced by SR-AIP, which, when normalized by load in CaMK/SR-AIP mice (Fig. 3c), results in a 50% decrease in leak. However, this may still not be a full recovery to the WT leak level, where SR Ca load is much higher than in CaMK mice (which by itself would increase leak).

Taken together, two independent techniques (tetracaine in intact cells and sparks in permeabilized cells) indicate that diastolic SR Ca^{2+} leak is reduced in CaMK/SR-AIP mice, but are probably not fully restored to WT levels.

Cell shortening and SR Ca^{2+} handling in intact myocytes

To test how overall myocyte Ca^{2+} homeostasis and function is altered we measured cell shortening and Ca^{2+} transients in fluo-4/AM loaded cardiomyocytes (representative traces in Fig. 4A–B). Fractional cell shortening was improved in cells expressing SR-AIP (%FS $1.33 \pm 0.22\%$ in CaMK vs $2.15 \pm 0.26\%$ in CaMK/SR-AIP, n=12 and 15 respectively, Fig. 4G +H), but there was no difference in the time to 50% relaxation (106 ± 9 ms in CaMK (n=13) vs. 95 ± 9 ms in CaMK/SR-AIP (n=16)) between groups. Consistent with the cell shortening data, the steady-state twitch Ca^{2+} transient amplitude at 1 Hz was increased in CaMK/SR-AIP mice ($\Delta F/F_0$ 2.85 ± 0.12 (n=35) compared to CaMK ($\Delta F/F_0$ 2.15 ± 0.16 (n=33); Fig. 4 C). Ca^{2+} transient amplitude was also significantly increased at lower (0.5Hz) and higher (2 Hz) stimulation frequency (data not shown). The Ca^{2+} transient amplitude-frequency response was similar in both groups (negative from 0.5–2 Hz, to $83.4 \pm 20\%$ in CaMK and to $89.6 \pm 23\%$ in CaMK/SR-AIP (n=17 and 15 respectively)). The time constant of exponential $[\text{Ca}^{2+}]_i$ decline τ (where 90% is dependent on SERCA function in mice [22]), was similar in both groups at all frequencies (shown for 1 Hz in Fig. 4D). Moreover, SR-AIP expression had no effect on the time to 50% relaxation and time-to-peak (data not shown). The Ca^{2+}

decline in the presence of caffeine is predominantly the result of the Na/Ca²⁺ exchanger (NCX) activity and the time constant τ was similar in both groups (Fig. 4F), indicating that the upregulation of NCX expression and function in CaMK vs WT mice [3] was unaltered by SR-AIP expression. Consistent with the observation that the Ca²⁺ transient amplitude is increased in intact, field stimulated myocytes from CaMK/SR-AIP group, SR Ca load was also increased, based on caffeine-induced Ca transients (Fig. 4E). In summary, SR Ca uptake and NCX function were not functionally altered by SR-AIP expression, consistent with the unaltered protein expression (see above), but the Ca²⁺ transient amplitude and the SR Ca²⁺ load were increased in CaMK/SR-AIP mice.

Ventricles are bigger, more dilated and in vivo cardiac function is worse

Despite the improvement in Ca²⁺ handling, the ventricular weight (VW) and VW divided by body weight (BW) were higher in CaMK/SR-AIP mice at 3 months of age (see table 2), indicating increased ventricular hypertrophy.

LV end-diastolic and end-systolic dimensions were also increased in CaMK and CaMK/SR-AIP when compared to WT (Fig 5A,B), indicative of ventricular dilation, which was also more severe in CaMK/SR-AIP. From the improvement in cardiac Ca²⁺ handling above we might expect less impairment of systolic function in the hearts of CaMK/ SR-AIP vs. CaMK, but fractional shortening (%FS; using M-mode echocardiography *in vivo*) was depressed in CaMK mice compared to either WT or mice expressing SR-AIP only (Fig 5C), and thus systolic dysfunction was further exacerbated in CaMK/SR-AIP mice. We also found that a higher number of CaMK/SR-AIP hearts had severely enlarged atria containing organized thrombi. Also, the only animal deaths in the group of mice set aside for echocardiography were in the CaMK/SR-AIP mice (2 of 9 mice), consistent with worsening prognosis when SR CaMKII is inhibited in CaMK mice. Since cellular function was not compromised and there was no evidence of cell death, the decrease in whole heart fractional shortening most likely is a consequence of the structural remodeling, largely due to ventricular dilation (law of Laplace: the larger the internal radius, the lower the developed pressure for a given wall tension). This indeed applies to dilated hearts as clinical studies have demonstrated that reducing left ventricular diameter of failing hearts improves systolic function [23,24].

Hypertrophy and interstitial fibrosis in young mice only in CaMK/SR-AIP

To directly confirm cellular hypertrophy, we measured cell size in PAS-stained sections. To test if the onset of hypertrophy is earlier in CaMK/SR-AIP mice we used sections from 1 month old mice. We found that cell diameter in LV and septum was already significantly increased in double-transgenic mice, but not in CaMK mice at this young age (Fig. 6D). There were also fewer nuclei per area (myocytes plus fibroblasts), likely due to the larger area occupied by hypertrophied myocytes (Fig. 6E). In addition, fibrosis was detected as blue staining in Masson's trichrome stained sections and analyzed as percent of area. The fibrotic area was increased in CaMK/SR-AIP mice, while CaMK mice were similar to control (Fig. 6F). No areas of replacement fibrosis were detected, consistent with the lack of local myocyte death, and indicative of interstitial fibrosis.

Discussion

SR function is altered in HF, with compromised SR Ca²⁺ uptake and increased SR Ca²⁺ leak, resulting in decreased pump function and increased risk for arrhythmia. CaMKII expression and activity are increased in HF [3,25–28] and this might contribute to the pathological phenotype. For example it has been shown in an arrhythmogenic rabbit HF model, that CaMKII inhibition, but not PKA inhibition, reduces the diastolic SR Ca²⁺ leak

to control values and restored SR Ca^{2+} content [3]. This particular study did not link the increase in leak with increased CaMKII phosphorylation of the RyR directly, but increased RyR activity after CaMKII phosphorylation has been observed in multiple studies [3,11,29]. Therefore CaMKII phosphorylation of the RyR is a prime candidate to contribute to the increase in SR Ca^{2+} leak.

Global inhibition of CaMKII by transgenic expression of the peptide AC3I has been shown to limit HF progression in response to chronic β -adrenergic activation [30]. Moreover, cardiac specific overexpression of CaMKII δ_C causes profound HF, diastolic SR Ca^{2+} leak, reduced SR Ca^{2+} content and Ca^{2+} transient amplitude, was accompanied by RyR phosphorylation at the known CaMKII site (S2814), and the leak enhancement (manifest as Ca^{2+} sparks) could be acutely inhibited by CaMKII inhibition [2,3]. On the other hand, genetic ablation of CaMKII δ limited the transition to HF upon aortic banding, in association with decreased SR Ca^{2+} leak and RyR phosphorylation at S2814 [5]. Thus, the RyR dysfunction caused by CaMKII appears to significantly contribute to depressed Ca^{2+} handling and HF progression. Restoring Ca^{2+} transient amplitude and SR Ca^{2+} content by crossing CaMKII δ_C transgenic mice with mice with PLB gene deletion exacerbated Ca^{2+} spark frequency in the CaMK mice and HF was worsened. There was evidence of greatly increased myocyte cell death in the crosses that suggested a causal role for the massive increase in Ca^{2+} spark frequency/ spark-mediated Ca^{2+} leak in mitochondrial Ca^{2+} loading and subsequent cardiomyocyte cell death [25]. Taking all these studies into consideration, it seemed highly reasonable to hypothesize that lowering the SR Ca^{2+} leak in the CaMK mice (and ultimately in failing hearts) would lower the SR Ca^{2+} leak and prevent HF. Our key observations are that SR-AIP reduced the maladaptive SR Ca^{2+} leak in CaMK mice by 20–50%, and accordingly enhanced SR Ca^{2+} content and Ca^{2+} transient amplitude in myocytes, but unexpectedly hypertrophy and cardiac dysfunction was worsened. There was no evidence for apoptotic cell death in the present study with CaMK/SR-AIP mice, perhaps not surprising since SR Ca leak was inhibited (rather than exacerbated as in the PLB-KO cross). Thus increased cell death is unlikely to explain the worsened whole heart function seen when SR-AIP is expressed in the CaMK mice. A more likely explanation is the increased ventricular dilation (due to a non-SR CaMKII target) and consequent mechanical disadvantage. Thus, while we could limit SR Ca^{2+} leak by targeted inhibition of CaMKII at the SR membrane using SR-AIP [4–6] and improve myocyte function, another non-SR CaMKII action may worsen the progression of hypertrophy and HF. Moreover, the improved global Ca transients might worsen this by enhancing the Ca^{2+} -dependent activation state of a non-SR CaMKII pool involved in this pathway.

Together this does not preclude a maladaptive role for SR leak induced effects (as suggested by studies cited above), and there are other known non-SR CaMKII pathways that have been implicated in hypertrophic signaling. This includes CaMKII-dependent phosphorylation of class II histone deacetylases (HDACs), which lead to HDAC nuclear export and de-repression of transcriptional regulation by MEF2 [31–33]. Our results also suggest, but don't prove that while SR-associated CaMKII is inhibited by SR-AIP, CaMKII activity in other locations may be enhanced (e.g. affecting nuclear transcription), which could contribute to the worsening hypertrophy and systolic dysfunction in CaMK/SR-AIP vs. CaMK.

Another consideration is that while we overexpress a particular isoform of CaMKII (δ_C), we can expect to inhibit all CaMKII isoforms present near the SR membrane and this may contribute to the worsening of the phenotype. E.g. a CaMKII β isoform that is targeted via α KAP to the SR membrane regulates local glycolytic enzymes with potential effects on subcellular NADH and ATP levels that may be detrimental in this setting [34].

SR-AIP and phosphorylation of PLB and RyR

Our primary goal in crossbreeding these mice was to inhibit the CaMKII dependent increase in diastolic Ca^{2+} leak, and this effort was successful. It is somewhat surprising that this was not accompanied by an inhibition of RyR-S2814 phosphorylation. We previously showed that SR-AIP reduces both PLB-T17 and RyR-S2814 phosphorylation when expressed in a WT background [6,8]. Here, when combined with CaMKII δ_C overexpression, SR-AIP could still inhibit PLB-T17 phosphorylation and microsomal CaMKII autophosphorylation, but it did not reduce RyR-S2814 phosphorylation (while the amount of RyR phosphorylated at S2814 was slightly reduced). However, the regulation of RyR phosphorylation is complex, and we and others have shown that more than one kinase phosphorylates RyR-PS2808 [35,36]. We recently demonstrated that at least one additional kinase in addition to CaMKII can phosphorylate the RyR-PS2814 site [35]. It also needs to be considered that yet unknown additional CaMKII phosphorylation sites may contribute to the increased leak in CaMK mice and that their phosphorylation is reduced in CaMK/SR-AIP mice [37].

SR-AIP has no direct effect on PKA activity, but PKA phosphorylation of PLB-S16 was increased and phosphorylation at RyR-S2808, also a PKA phosphorylation site, was decreased. It is not obvious how these two changes relate to CaMKII effects, and CaMK mice did not show altered PLB-S16 phosphorylation vs. WT [2] while RyR-S2808 was increased in CaMK mice. So the reduction in RyR phosphorylation could be part of a local reversion of HF phenotype at the SR, but the potent increase of PLB phosphorylation might suggest some hyper-adrenergic state (or possibly altered local phosphatase activity not detected by our assessment of total phosphatase expression).

We conclude that inhibition of CaMKII by SR-AIP in CaMKII δ_C overexpressing mice improves myocyte Ca^{2+} handling, but does not rescue the contractile dysfunction associated with heart failure. This implies that CaMKII δ_C exerts SR-independent effects that contribute to hypertrophy and HF. We also speculate that in pathological conditions, when maladaptive CaMKII signaling is increased, diminished SR Ca^{2+} load and release may be beneficial. Further work will be required to test whether specific local inhibition of CaMKII can become a novel, targeted therapeutic approach in HF without compromising physiological function.

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Figure 1. Transgene expression and phosphorylation

Transgene expression and phosphorylation in CaMK and CaMK/SR-AIP mice. (A) In CaMK mice, PLB migrates predominantly as a pentamer at about 20 kDa (unboiled samples), but in CaMK/SR-AIP mice also higher molecular weight bands are observed. Those form due to oligomerization with the larger SR-AIP. (B,C) CaMKII δ_C overexpression is similar in both groups. Due to the low amount of protein loaded other endogenous isoforms are not observed (e.g. CaMKII δ_B). (D) CaMKII δ_C autophosphorylation and abundance in microsomal tissue fraction. (E) CaMKII δ_C PT287 autophosphorylation in microsomal fraction and total heart homogenate. * $p \leq 0.05$ vs CaMK

Figure 2. Expression and Phosphorylation of Ca²⁺ handling proteins

Protein expression and phosphorylation of PLB (A,B) and RyR (C–E) measured by Western blot in whole heart homogenates (all n=6–8). Inserts above graphs are representative Western blots. (B,E) show phosphorylation levels relative to the expression level, but (D) shows total amount of phosphorylated RyR. All data are reported relative to previously published data comparing WT vs CaMK (with the exception of (D) and PS2808 which has not been reported before). * p ≤ 0.05 vs CaMK

Figure 3. Decreased diastolic Ca²⁺ leak

Quantitative assessment of diastolic Ca²⁺ leak using tetracaine. (A) Representative tracings. The leak is determined by the increase in diastolic free Ca²⁺ after removal of tetracaine. (B) Summary of results (n=17–20). The tetracaine-sensitive Ca leak is plotted versus the SR Ca²⁺ load measured by caffeine application. Although the SR Ca²⁺ load is increased in CaMK/SR-AIP, the SR Ca²⁺ leak is reduced. (C) The arbitrary leak/load ratio is reduced in CaMK/SR-AIP myocytes. * p ≤ 0.05 vs CaMK

Figure 4. Increased Ca²⁺ transients, SR Ca²⁺ load and cell shortening

Ca²⁺ homeostasis and cell shortening in intact myocytes. (A,B) Representative tracings of cells that were field stimulated at 1 Hz. (C) The Ca²⁺ transient amplitude expressed as $\Delta F/F_0$ is increased. (D) The time constant τ , determined by fitting the Ca²⁺ transient decline monoexponentially, yields information about SERCA uptake function and was not altered in CaMK/SR-AIP myocytes. (E) The amount of Ca²⁺ released from the SR upon rapid application of caffeine is higher in CaMK/SR-AIP myocytes. (F) The decline of the caffeine transient in the presence of caffeine, also fitted monoexponentially, yields information about NCX function and is also similar between groups. (G) Fractional shortening in % of cell

length (%FS) measured via edge-detection was higher in CaMK/SR-AIP group, while (H) time to 50% relaxation was unaltered. * $p \leq 0.05$ vs CaMK

Figure 5. *In vivo* M-mode echocardiography

Heart dimensions (left ventricular (LV) end-diastolic dimension (LVEDD, A) and LV end-systolic dimension (LVESD, B)) and the percentage of LV fractional shortening (%FS, C) was determined in anesthetized mice using standard M-mode echocardiography. All four groups were evaluated, including wild-type (WT) and transgenic mice expressing SR-AIP only (SR-AIP) (columns both striped). All parameters were significantly different between WT vs CaMK and CaMK/SR-AIP (*), but not between WT and SR-AIP. CaMK/SR-AIP showed increased LV dimensions and decreased %FS when compared to CaMK (#).

Figure 6. Earlier Onset of Hypertrophy and Remodeling Histology in one month old mice
 Histological analyses of heart sections from one month old mice. Representative PAS-stained sections from WT (A), CaMK (B) and CaMK/SR-AIP mice (C) that were used for analysis of cell diameter (D) and counting of nuclei using image thresholding (E). The fibrosis content was analyzed from Masson's trichrome stained sections by image color thresholding (F). Five images were taken from the left ventricle of four hearts per group. Cell diameter was measured in 56-91 cells per image by placing a line across a myocyte with clearly visible cell border at the level of the nucleus (minimum of 1438 cells per group). Cell size and fibrosis content were increased in CaMK/SR-AIP group at an age when CaMK mice were not significantly different from WT mice.

Table 1

Spark properties and SR load in permeabilized cells

20% brightest sparks	CaMK	CaMK/SR-AIP	Unit
<i># Sparks analyzed</i>	990	1015	
<i>Frequency</i>	6.90±0.23	6.58±0.36	sparks/100µm/s
<i>Amplitude</i>	1.945±0.016	1.913±0.013	ΔF/F ₀
<i>Signal mass</i>	37.4±2.0	30.0±1.9*	µm ³
<i>FWHM</i>	2.28±0.02	2.04±0.03*	µm
<i>FDHM</i>	36.1±0.5	31.4±0.4*	ms
<i>full Width</i>	4.79±0.047	4.21±0.049*	µm
<i>full Duration</i>	86.0±1.3	75.4±1.2*	ms
<i>Time-to-peak</i>	20.7±0.43	17.4±0.33*	ms
<i>Upstroke</i>	173±1.9	178±1.8*	Δ(F/F ₀)/Δt max
<i>Tau</i>	38.8±0.77	32.4±0.59*	ms
SR load			
<i>Caffeine</i>	3.62±0.25	3.77±0.20	ΔF/F ₀

Table 2

Gross observations

Gross observations	CaMK (n=8–11)	CaMK/SR-AIP (n=13)
<i>Age (days)</i>	97±5	86±4
<i>Ventricular weight (VW)</i>	276±24	365±20*
<i>Body weight (BW)</i>	27.0±1.3	27.4±1.1
<i>VW/BW ratio</i>	10.2±0.8	13.5±0.9*
<i>Enlarged atria</i>	45%	84%