Phospholamban Ablation Rescues Sarcoplasmic Reticulum Ca\(^{2+}\) Handling but Exacerbates Cardiac Dysfunction in CaMKI\(\delta\) Transgenic Mice

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Rationale: We previously showed that transgenic mice expressing Ca\(^{2+}\)/calmodulin-dependent protein kinase II \(\delta\) (CaMKII-TG) develop dilated cardiomyopathy associated with increased ryanodine receptors (RyR2) phosphorylation, enhanced sarcoplasmic reticulum (SR) Ca\(^{2+}\) leak and lowering of SR Ca\(^{2+}\) load. We hypothesized that phospholamban (PLN) ablation would restore SR Ca\(^{2+}\) load and prevent the decreased ventricular contractility, dilation and mortality seen in CaMKII-TG.

Objective: Our objectives were to generate CaMKII-TG mice lacking PLN, determine whether the maladaptive effects of cardiac CaMKII\(\delta\) expression were corrected, and establish the mechanistic basis for these changes.

Methods and Results: CaMKII-TG were crossed with PLN knockout (PLN-KO) mice to generate KO/TG mice. Myocytes from wild type (WT), CaMKII-TG, PLN-KO and KO/TG were compared. The decreased SR Ca\(^{2+}\) load and twitch Ca\(^{2+}\) transients seen in CaMKII-TG were normalized in KO/TG. Surprisingly the heart failure phenotype was exacerbated, as indicated by increased left ventricular dilation, decreased ventricular function, increased apoptosis and greater mortality. In KO/TG myocytes SR Ca\(^{2+}\) sparks and leak were significantly increased, presumably because of the combined effects of restored SR Ca\(^{2+}\) load and RyR2 phosphorylation. Mitochondrial Ca\(^{2+}\) loading was increased in cardiomyocytes from KO/TG versus WT or CaMKII-TG mice and this was dependent on elevated SR Ca\(^{2+}\) sparks. Cardiomyocytes from KO/TG showed poor viability, improved by inhibiting SR Ca\(^{2+}\) release and mitochondrial Ca\(^{2+}\) loading.

Conclusions: Normalizing cardiomyocyte SR Ca\(^{2+}\) loading in the face of elevated CaMKII and RyR2 phosphorylation leads to enhanced SR Ca\(^{2+}\) leak and mitochondrial Ca\(^{2+}\) elevation, associated with exacerbated cell death, heart failure and mortality. (Circ Res. 2010;106:354-362.)

Key Words: calcium ■ Ca\(^{2+}\)/calmodulin-dependent protein kinase II ■ phospholamban ■ heart failure

Ca\(^{2+}\) is a critical second messenger in cardiac function. The Ca\(^{2+}\)/calmodulin-dependent protein kinase (CaMK)II is regulated by and involved in control of Ca\(^{2+}\) cycling in the myocardium. Several years ago we generated transgenic mice expressing CaMKI\(\delta\), the predominant cardiac CaMK isoform. Mice expressing the CaMKI\(\delta\)-splice variant in the myocardium (CaMKII-TG) develop heart failure (HF) associated with ventricular dilation, marked decreases in fractional shortening, and mortality.1 We established that there was increased phosphorylation of the cardiac ryanodine receptor (RyR2) at the CaMKII site, associated with increased sarcoplasmic reticulum (SR) Ca\(^{2+}\) spark frequency and increased diastolic SR Ca\(^{2+}\) leak. More recently we generated CaMKI\(\delta\) knockout mice and further demonstrated that HF induced by pressure overload was dependent on CaMKI\(\delta\)-mediated RyR2 phosphorylation and increased Ca\(^{2+}\) sparks.2

The increased diastolic Ca\(^{2+}\) leak seen in the CaMKII-TG resulted in profound SR Ca\(^{2+}\) depletion and reduced Ca\(^{2+}\) transients and contractions. Accordingly, we hypothesized that diminished SR Ca\(^{2+}\) load could be responsible for the decreased contractile function and concomitant morbidity that characterized these mice. Cardiac SR Ca\(^{2+}\) uptake is regulated through the interaction of phospholamban (PLN) with the SR Ca\(^{2+}\) ATPase (SERCA2a).3 PLN knockout mice (PLN-KO) exhibit enhanced myocardial contractility,4 which

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suggest that interventions that increase SR Ca\textsuperscript{2+} in the face of enhanced diastolic Ca\textsuperscript{2+} leak predispose to cardiomyocyte cell death and likely other Ca\textsuperscript{2+} mediated toxicities that compromise survival.

Methods

Mice

CaMKII-TG mice\textsuperscript{1} were crossed with PLN-KO mice\textsuperscript{4} and heterozygous PLN offspring carrying the CaMKII transgene inbred with the ones without the transgene. Four genotypes of mice including wild type (WT), PLN-KO, CaMKII-TG, and PLN ablation with CaMKII\textsuperscript{0/0} overexpression (KO/TG) were used for experiments. All mice used in the present study were of mixed gender (more male than female) at 8 weeks of age, unless otherwise noted. All procedures were performed in accordance with NIH Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee.

Ca\textsuperscript{2+} Measurements of Adult Mouse Ventricular Myocytes

Mouse ventricular myocytes were isolated and loaded with indo-1-AM to assess SR Ca\textsuperscript{2+} content and twitch [Ca\textsuperscript{2+}],\textsuperscript{transients}, and with fluo-4-AM for SR Ca\textsuperscript{2+} spark measurements as described previously.\textsuperscript{9} Ca\textsuperscript{2+} sparks were measured in intact myocytes and were evaluated as described (IDL 5.3 computer software).\textsuperscript{9} Ca\textsuperscript{2+} spark amplitudes were normalized (F\textsubscript{F}0) to fluorescence baseline (F\textsubscript{F}). Duration of the Ca\textsuperscript{2+} sparks was taken as the full duration above half-maximum (FDHM) and width was the full spatial size above half maximum (FWHM). A crude index of diastolic SR Ca\textsuperscript{2+} leak was calculated as Ca\textsuperscript{2+} spark frequency (CaSpF)×FDHM×FWHM/amplitude. Some myocytes were preincubated for 30 minutes with a cell-permeable myristoylated CaMKII inhibitor, autacam tide-2 related inhibitory peptide II (AIP) (Calbiochem no. 189485), before measurements.

Mitochondrial Ca\textsuperscript{2+} Measurements of Adult Mouse Ventricular Myocyte

Mitochondrial Ca\textsuperscript{2+} was measured indirectly by examining the carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)-induced increase in [Ca\textsuperscript{2+}], in myocytes loaded with 10 \mu mol/L fura-2-AM (Molecular Probes; 30 minutes plus 10 minutes of deesterification). Fura-2 was excited at 340±10 and 380±10 nm, and emitted fluorescence (535±20 nm) was recorded at 100 Hz. Background-subtracted ratio fluorescence (R=F\textsubscript{380}/F\textsubscript{340}) was converted to [Ca\textsuperscript{2+}], using [Ca\textsuperscript{2+}]=K\textsubscript{d}β(R-R\textsubscript{min})/(R\textsubscript{max}−R). R\textsubscript{min}, R\textsubscript{max}, K\textsubscript{d} and β were determined experimentally. To assess baseline SR Ca\textsuperscript{2+} content myocytes were first paced at 0.5 Hz to steady state, and 10 mM/L caffeine was rapidly applied. After caffeine washout, myocytes were paced at 0.5 Hz to reattain the same steady state. They were then exposed to 0Na\textsuperscript{+}/0Ca\textsuperscript{2+}/0K\textsuperscript{+} solution to block the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (NCX) and Na\textsuperscript{+}/K\textsuperscript{+} pump and treated with 10 \mu mol/L thapsigargin for ~45 seconds to block the SERCA pump.\textsuperscript{10} Under these conditions, when 1 \mu mol/L FCCP is applied to abolish the mitochondrial membrane potential, mitochondrial Ca\textsuperscript{2+} is released and the rise in [Ca\textsuperscript{2+}] is indicative of mitochondrial Ca\textsuperscript{2+} content. Oligomycin (1 \mu mol/L) was included with FCCP to block rapid ATP consumption. SR Ca\textsuperscript{2+} load assessed by application of 10 \mu mol/L caffeine at the end of the protocol showed that the SR was still well Ca\textsuperscript{2+}-loaded (and subsequent relaxation and [Ca\textsuperscript{2+}] decline) confirm that ATP was not dissipated by the protocol. The SR cannot be reloading after this depletion, confirming the thapsigargin-dependent SR Ca\textsuperscript{2+}-ATPase inhibition.\textsuperscript{10}

Transthoracic Echocardiography

Transthoracic echocardiography was performed in mice using an Agilent Technologies Sonos 5500 system with a 15 MHz transducer as described.\textsuperscript{1}
Western Blotting
Western blot analysis was carried out in cardiac homogenates as described previously. The antibodies used for immunoblotting were as follows: rabbit anti-calsequestrin, rabbit anti-SERCA2, mouse anti-RyR, mouse anti–phospho-CaMKII (Affinity Bioreagents), rabbit Ser2809 phospho-RyR2 antibody (Badrilla, Leeds, UK), rabbit Ser2815 phospho-RyR2 antibody (a gift from A. R. Marks, Columbia University, New York), and anti-NCX (monoclonal R3F1, a gift from K. D. Philipson, University of California at Los Angeles).

TUNEL Staining
Transverse sections of mouse hearts were labeled with fluorescein-TUNEL using a kit from Roche (Lewes, UK) according to the instructions of the manufacturer. Slides were mounted with Vectashield mounting media containing DAPI to stain for the nuclei. Wheat germ agglutinin was used to stain cell membranes and cardiomyocytes identified by their centrally located nuclei. Labeled nuclei were counted to determine the apoptotic index (number of labeled nuclei/105 total myocyte nuclei).

Cell Viability Study in Adult Mouse Ventricular Myocytes
Ventricular myocytes were isolated as described previously. After isolation, cells were plated for 1 hour on laminin-coated dishes in MEM-HBSS (Hanks balanced salt solutions) medium containing 5% serum. Cells were then washed, and serum-free medium added along with vehicle, 2 μmol/L KN-93, 100 nmol/L ryanodine, 5 μmol/L Ru-360, or 5 μmol/L cyclosporin A. Using a grid marking system, 8 fields were chosen per dish and the number of rod shaped living cells was counted at various times over a 12-hour time course. At the 12-hour time point, living cells were quantitated by trypan blue exclusion.

Statistical Analysis
All data are reported as means±SEM. Statistical significance of differences between groups was determined using 1-way ANOVA with Tukey post hoc test. A probability value of <0.05 was considered statistically significant.

Results

SR Ca2+ Load and Ca2+ Transients in Isolated Cardiomyocytes
To determine whether PLN ablation normalized the depressed SR Ca2+ load observed in the CaMKIIε TG mice, SR Ca2+ content was assessed by quantifying caffeine-induced Ca2+ transients after 1 Hz steady-state stimulation (Figure 1A). Consistent with our previous reports, myocytes isolated from PLN-KO showed increased SR Ca2+ load, whereas those from CaMKII-TG mice exhibited significantly depressed SR Ca2+ load (Figure 1A and 1B). SR Ca2+ load was restored to levels equivalent to those of WT mice in myocytes from the KO/TG crosses (Figure 1A and 1B). NCX function, assessed as the rate constant of [Ca2+]i decline (kCa) in the presence of caffeine, was significantly faster in CaMKII-TG versus WT as shown previously, and PLN ablation did not normalize this difference in NCX function (Figure 1C). Twitch Ca2+ transient amplitude (Δ[Ca2+]i, at 1 Hz) was also restored in KO/TG versus CaMKII-TG myocytes (Figure 2). In fact, Ca2+ transients in KO/TG were greater than those in WT, likely reflecting the combination of restored SR Ca2+ content along with CaMKII-dependent enhancement of fractional SR Ca2+ release.

In Vivo Global Cardiac Function
To determine whether the restoration of myocyte Ca2+ handling was associated with a diminished hypertrophic/HF phenotype and improved hemodynamic function, we measured the heart/body weight (HW/BW) ratio and assessed in vivo cardiac diameters and function by echocardiography. As reported previously, CaMKII-TG mice showed significant cardiac enlargement at 8 weeks of age as assessed by HW/BW ratio; surprisingly, the increase in HW/BW was even greater in KO/TG mice (Figure 3A), as was cell size (not

Figure 1. PLN ablation in CaMKIIε TG mice repletes SR Ca2+ load in isolated cardiomyocytes. A, Representative caffeine-induced Ca2+ transient traces. B, Average Δ[Ca2+]i for caffeine-induced Ca2+ transients (SR Ca2+ load). *P<0.05 vs WT. #P<0.05 vs CaMKII-TG. C, Average rate constant kCa for [Ca2+]i decline during caffeine exposure (which indicates NCX activity). *P<0.05 vs WT; **P<0.01 vs WT. The number of cells studied: WT n=6, PLN-KO n=21, CaMKII-TG n=9, and KO/TG n=9.
shown). Left ventricular dilation and cardiac dysfunction were also worsened in KO/TG mice relative to CaMKII-TG, as revealed by significantly greater left ventricular end diastolic dimension, diminished fractional shortening, and posterior wall thinning at 6 to 8 weeks (Figure 3B and 3C; Online Figure I, available in the Online Data Supplement at http://circres.ahajournals.org). Differences between KO/TG and CaMKII-TG mice were most significant at earlier stages (4 to 5 weeks), becoming less divergent at 12 to 16 weeks (Figure 3B and 3C, lower panels; Online Figure I).

**Survival Analyses**

As described previously,1 CaMKIIΔc TG mice showed premature death. KO/TG mice demonstrated worsened mortality compared to CaMKII-TG mice, with 70% of the KO/TG mice dying by 24 weeks of age (Figure 3D).

**Expression and Phosphorylation of Ca2+ Regulatory Proteins**

Western blot analyses were performed to assess the expression levels of calsequestrin, SERCA2a and NCX in ventricular homogenates from CaMKII-TG and KO/TG mice. As previously reported,9 in CaMKII-TG versus WT, we found reduced SERCA2a and elevated NCX expression but unaltered calsequestrin expression (data not shown). There were no significant differences in the expression of these major Ca2+ regulatory proteins in KO/TG versus CaMKII-TG mice (Figure 4A). Phosphorylation of the RyR2, assessed using antibodies to the CaMKII phosphorylation sites (Ser2815 and Ser2809), was increased significantly, but not differentially, in CaMKII-TG and KO/TG mice (Figure 4B). These data indicate that the exaggerated phenotype observed in KO/TG mice is not secondary to changes in key Ca2+ handling proteins or greater RyR2 phosphorylation.

**SR Ca2+ Sparks and Ca2+ Leak in Isolated Cardiomyocytes**

We previously demonstrated increased Ca2+ spark frequency in CaMKII-TG versus WT myocytes.9 To determine whether

![Figure 2. PLN ablation in CaMKIIΔc TG mice normalizes twitch Ca2+ transients in isolated cardiomyocytes. A, Representative twitch [Ca2+]i transient traces. B, Average Δ[Ca2+]i for twitch-induced Ca2+ transients. *P<0.05 vs WT; #P<0.05 vs CaMKII-TG. The number of cells studied: WT n=6, PLN-KO n=21, CaMKII-TG n=9, and KO/TG n=9.](image-url)

![Figure 3. PLN ablation in CaMKIIΔc TG mice exaggerates the phenotype of dilated cardiomyopathy. A, Heart weight/body weight (HW/BW) ratio at 8 weeks. B and C, Averaged echocardiographic parameters at different ages. Data are presented for B, left ventricular end-diastolic diameter (LVEDD), and for C, calculated percent fractional shortening (FS). n=4 to 6 mice per group at each age; *P<0.05 vs WT. #P<0.05 vs CaMKII-TG. D, Survival curves for CaMKII-TG and PLN-KO/CaMKII-TG mice. PLN ablation accentuates premature death in CaMKIIΔc TG mice. Numbers of mice were as follows: CaMKII-TG, n=16; KO/TG, n=22. P<0.01 KO/TG vs CaMKII-TG.](image-url)
the enhanced SR Ca\(^{2+}\) content seen with PLN ablation was
associated with a further increase in SR Ca\(^{2+}\) sparks and leak,
Ca\(^{2+}\) spark frequency (CaSpF), duration (FDHM), spatial width (FWHM), and amplitude were assessed. CaSpF increased dramatically in KO/TG compared to CaMKII-TG. This was entirely blocked by pretreatment with a CaMKII inhibitory peptide, AIP (Figure 5A), indicating that it resulted from ongoing CaMKII activity. The overall diastolic SR Ca\(^{2+}\) leak index (CaSpF x FDHM x FWHM x amplitude) was also significantly increased in KO/TG mice (7.5-fold relative to WT and 2.5-fold relative to CaMKII-TG) (Figure 5B). The augmented SR Ca\(^{2+}\) sparks and leak in KO/TG crosses can be explained in terms of the combined effects of normalized SR Ca\(^{2+}\) load in the face of enhanced RyR2 phosphorylation. Notably enhanced SR Ca\(^{2+}\) loading, Ca\(^{2+}\) spark frequency, and Ca\(^{2+}\) transients are observed in KO/TG myocytes despite lower diastolic [Ca\(^{2+}\)] levels measured under comparable conditions (Online Figure II).

Apoptosis in Heart Sections
Cardiomyocyte dropout could explain why there is reduced cardiac function, ventricular thinning and worsened HF despite enhanced Ca\(^{2+}\) transients in KO/TG myocytes. TUNEL staining was performed to assess apoptosis in paraffin sections prepared from WT, PLN-KO, CaMKII-TG, and KO/TG mice. DAPI staining and wheat germ agglutinin were used to identify cardiomyocytes (see Methods). TUNEL-labeled cardiomyocyte nuclei were counted to determine the apoptotic index (number of labeled nuclei/10\(^5\) total myocyte nuclei). As shown in Figure 6, the number of TUNEL-positive cardiomyocytes was significantly increased (~2.6 fold) in KO/TG mouse hearts compared with WT or CaMKII-TG mice.

Cell Viability Studies
The assessment of TUNEL staining captures a single time point but suggests that the viability of cardiomyocytes is compromised in the KO/TG line. To further examine cell viability, we isolated and monitored cells by morphology and trypan blue exclusion at various times (Figure 7). To test whether CaMKII or SR Ca\(^{2+}\) release were important in regulating cell viability, cells were pretreated with 2 \(\mu\)mol/L KN-93 (to inhibit CaMKII) or 100 nmol/L ryanodine (to

Figure 4. Quantitative immunoblotting of major Ca\(^{2+}\) handling proteins in mouse ventricular homogenates. A, Expression levels of calsequestrin (CsQ), SERCA, and NCX did not change significantly between the CaMKII-TG and the PLN-KO/CaMKII-TG. B, Phosphorylation of ryanodine receptors (RyR2) at Ser2809 and Ser2815 (normalized to total RyR2) was increased significantly but not differentially in CaMKII-TG and PLN-KO/CaMKII-TG mice. *P<0.05 vs WT; n=4 mice per group.

Figure 5. Enhanced SR Ca\(^{2+}\) spark frequency and Ca\(^{2+}\) leak in intact cardiomyocytes from PLN-KO/CaMKII-TG mice. A, SR Ca\(^{2+}\) spark frequency (CaSpF). *P<0.05 vs WT; #P<0.05 vs CaMKII-TG; +P<0.05 vs KO/TG. B, SR Ca\(^{2+}\) leak. *P<0.05 vs WT; #P<0.05 vs CaMKII-TG; +P<0.05 vs KO/TG.
deplete SR Ca$^{2+}$ and prevent Ca$^{2+}$ sparks). WT myocytes remained viable over a 12-hour period, and survival was not appreciably altered by KN-93 or ryanodine (Figure 7A). In contrast, the percentage of live myocytes from both CaMKII-TG and KO/TG mice decreased dramatically over 12 hours (to less than 30%). Inhibition of CaMKII with KN-93 or of SR Ca$^{2+}$ leak with ryanodine decreased the rate of myocyte death in both CaMKII-TG and KO/TG mice (Figure 7B and 7C), suggesting that the CaMKII-mediated increase in SR Ca$^{2+}$ release contributes to diminished myocyte viability.

**Mitochondrial Ca$^{2+}$ in Isolated Cardiomyocytes**

The dramatic increase in diastolic Ca$^{2+}$ sparks could increase mitochondrial Ca$^{2+}$ loading and thereby contribute to decreased cardiomyocyte viability. To explore this possibility, we assessed mitochondrial Ca$^{2+}$ content in isolated adult myocytes from WT, CaMKII-TG, or KO/TG mice as detailed in Methods (Figure 8A). The protocol was designed to allow mitochondrial Ca$^{2+}$ loading to occur during regular pulses (see initial cytosolic [Ca$^{2+}$]$_i$ transients) and a 40-second rest period where Ca$^{2+}$ sparks could occur. The release of mitochondrial Ca$^{2+}$ was then induced using FCCP, under conditions where the released Ca$^{2+}$ is trapped in the cytosol (SERCA, NCX, and Na$^+$/K$^+$.ATPase inhibited) and quantitated by the rise in [Ca$^{2+}$]$_i$. Figure 8A demonstrates that the FCCP-induced rise in [Ca$^{2+}$]$_i$ in KO/TG (black trace) is substantially larger than that in WT (gray trace), reflecting a greater mitochondrial Ca$^{2+}$ load. Mitochondrial Ca$^{2+}$ load was more than 4-fold higher in KO/TG versus WT or CaMKII-TG myocytes (Figure 8B).

To more directly test the relationship between Ca$^{2+}$ sparks and mitochondrial Ca$^{2+}$ loading in KO/TG myocytes, external Ca$^{2+}$ was reduced (to 0.3 mmol/L) and CaMKII was inhibited (with AIP). This restored SR Ca$^{2+}$ content and Ca$^{2+}$ transients (Figure 8C), as well as Ca$^{2+}$ spark frequency (not shown) in KO/TG myocytes to near WT levels. Strikingly, mitochondrial Ca$^{2+}$ was not increased in KO/TG myocytes under these conditions (Figure 8B).

The role of mitochondrial Ca$^{2+}$ overloading in myocyte viability was examined by treating cells with an inhibitor of the mitochondrial uniporter (Ru-360) or the mitochondrial permeability transition (PT)-pore (cyclosporin A). Both agents significantly enhanced survival of KO/TG cardiomyocytes (Figure 8D), implicating mitochondrial Ca$^{2+}$ overloading and the PT-pore in the diminished viability of these versus WT cells. On the other hand, neither agent increased the survival of CaMKII-TG myocytes (Figure 8D), suggesting that mitochondrial Ca$^{2+}$ overloading is less critical to the viability of the CaMKII-TG cells.

**Discussion**

**PLN As a Target for HF Treatment**

The SR Ca$^{2+}$ content of cardiomyocytes is a key determinant of SR Ca$^{2+}$ release and resultant Ca$^{2+}$ transients. SR Ca$^{2+}$ uptake is decreased in many animal models of HF and human failing heart, and this has been suggested to cause contractile dysfunction by decreasing the stored Ca$^{2+}$ available for
release. Ca\(^{2+}\) uptake into the SR is mediated by the SR Ca\(^{2+}\) ATPase (SERCA2a) and regulated by PLN, an endogenous inhibitor of SERCA2a whose activity is regulated by phosphorylation. Ablation or inhibition of PLN has been proposed as a viable therapeutic strategy for treating HF by restoring SR Ca\(^{2+}\) load\(^{14,15}\); this notion is supported by a number of reports, including the rescue of cardiomyopathy and HF in muscle LIM protein KO\(^6\) and calsequestrin or \(^{125}\) transgenic mice\(^{7,8}\) and the suppression of HF progression in cardiomyopathic hamsters\(^{16}\) and in rats following myocardial infarction\(^{17}\). In sharp contrast, in the present study, as in several other genetic hypertrophy and HF models\(^{18,19}\), PLN ablation clearly restored myocyte Ca\(^{2+}\) stores, Ca\(^{2+}\) transients and contraction, but failed to rescue in vivo cardiac function or the HF phenotype. Thus, although depressed SR Ca\(^{2+}\) uptake is an important feature of HF (and a target to correct cellular Ca\(^{2+}\) dysregulation), restoration of SR Ca\(^{2+}\) handling does not appear to rescue all sequelae associated with HF development. The dissociation between improved cardiomyocyte SR Ca\(^{2+}\) handling and the in vivo loss of ventricular function and survival in the KO/TG mice provided an opportunity to explore, at the cellular level, why restoring SR Ca\(^{2+}\) content fails to improve, and indeed may worsen, global cardiac function.

\textbf{Ca\(^{2+}\), Mitochondria, and Cell Death}\n
SR Ca\(^{2+}\) sparks, elementary events of diastolic SR Ca\(^{2+}\) release, have been reported to be enhanced by both CaMKII overexpression and PLN ablation\(^9,20\). The CaMKII effect is primarily attributable to an activating effect on RyR2\(^12\), whereas PLN-KO enhances Ca\(^{2+}\) sparks indirectly by increasing SR Ca\(^{2+}\) content, further enhancing RyR2 opening resulting from a regulatory effect of intra-SR [Ca\(^{2+}\)]\(^{12,21}\). Here, we demonstrate that SR Ca\(^{2+}\) sparks are massively increased in the KO/TG myocytes because of the combination of CaMKII mediated RyR2 phosphorylation and elevated SR Ca\(^{2+}\) load.

Numerous studies have demonstrated that mitochondrial Ca\(^{2+}\) overload results in opening of the permeability transition (PT)-pore and that this leads to either apoptotic or necrotic cell death in various cell types including cardiomyocytes.\(^{22-27}\) Cardiomyocyte loss by apoptosis has been recognized as a major factor contributing to HF development.\(^{28,29}\) Necrosis also contributes to ventricular remodeling induced by ischemic damage or in HF.\(^{28,29,31}\) Enhancement of cardiomyocyte Ca\(^{2+}\) influx, as observed in transgenic mice expressing the sarcolemmal L-type Ca\(^{2+}\) channel, is one mechanism for eliciting Ca\(^{2+}\) overload, mitochondrial PT-pore opening and necrotic myocyte death.\(^{32}\) Mitochondria take up cytosolic Ca\(^{2+}\) via a Ca\(^{2+}\)-uniporter and a close relationship between SR and mitochondria, in which Ca\(^{2+}\) released from the SR is efficiently transmitted to the mitochondria in cardiomyocytes, has been reported.\(^{33}\) We suggest that the dramatic increase in Ca\(^{2+}\) sparks in the KO/TG myocyte leads, either directly or indirectly, to increased mitochondrial Ca\(^{2+}\) loading, PT-pore

\textbf{Figure 8.} Involvement of mitochondrial Ca\(^{2+}\) in myocyte death in PLN-KO/CaMKII-TG mice. A, Mitochondrial Ca\(^{2+}\) was measured as shown in the protocol. NT indicates no treatment; Thaps, thapsigargin. B, Mitochondrial Ca\(^{2+}\) was increased in cardiomyocytes isolated from PLN-KO/CaMKII-TG mice. \(*P<0.05\) vs WT. Mitochondrial Ca\(^{2+}\) in PLN-KO/CaMKII-TG myocytes returned to control levels with reduced external Ca\(^{2+}\) (0.3 mmol/L) and CaMKII inhibitor AIP. C, Reduced external Ca\(^{2+}\) and CaMKII inhibition with AIP returned SR Ca\(^{2+}\) content and Ca\(^{2+}\) transients to near control myocyte levels. D, Inhibition of the mitochondrial uniporter by Ru-360 or inhibition of the mitochondrial permeability transition pore by cyclosporin A decreased the rate of death in isolated cardiomyocytes from PLN-KO/CaMKII-TG mice at 12 hours. \(*P<0.05\) vs WT.
opening, and subsequent cardiomyocyte loss. This mechanism is supported by data demonstrating that mitochondrial Ca\(^{2+}\) loading and cell death are diminished following inhibition of Ca\(^{2+}\) sparks or mitochondrial Ca\(^{2+}\) uptake. Loss of functional cardiomyocytes could explain why the KO/TG hearts perform more poorly than the CaMKII-TG heart, despite improved cardiomyocyte Ca\(^{2+}\) transients.

**Sympathetic Tone, CaMKII, and HF**

In HF, there is typically increased sympathetic tone and PKA activation,\(^4,34\) as well as increased expression and activation of CaMKII\(^14-37\) and enhanced RyR2 phosphorylation and SR Ca\(^{2+}\) leak.\(^34,38\) Under these conditions, where phosphorylated RyR2 may already enhance diastolic SR Ca\(^{2+}\) release, increased adrenergic signaling or therapeutic attempts to replete SR Ca\(^{2+}\) stores could exacerbate increased SR Ca\(^{2+}\) leak (as with PLN ablation). As suggested above, the increased leak could trigger mitochondrial Ca\(^{2+}\) overloading with concomitant cardiomyocyte death.\(^27,32\) This may also help to explain data demonstrating that CaMKII activation contributes to cardiomyocyte cell death induced by \(\beta\)-adrenergic stimulation and to isoproterenol-induced apoptosis or cardiomyopathy in vivo.\(^39-41\)

Increased SR Ca\(^{2+}\) leak induced by concomitant increases in SR Ca\(^{2+}\) content and RyR2 phosphorylation could also predispose to arrhythmias, a possible basis for the observed increase in mortality in the KO/TG mice. Although arrhythmias may not occur in all forms of HF in which SERCA function is impaired,\(^26\) increased diastolic SR Ca\(^{2+}\) loading and cell death. This data are consistent with the working hypothesis that in the face of phosphorylation-activated RyR2 channels, repletion of Ca\(^{2+}\) stores through PLN ablation (or during sympathetic activation) can exacerbate SR Ca\(^{2+}\) leak and thereby increase mitochondrial Ca\(^{2+}\) -mediated cell death or activate other Ca\(^{2+}\)-dependent processes that contribute to cardiac dysfunction.

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**Disclosures**

None.

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Online Figure I. Averaged left ventricular posterior wall (LVPW) thickness determined by echocardiography at different ages. LV posterior wall thinning was greater in PLN-KO/CaMKII-TG mice vs. WT at all ages (and even vs. CaMKII-TG at early ages). *$P<0.05$ vs. WT.
Online Figure II. Averaged diastolic [Ca\textsuperscript{2+}]\textsubscript{i} in isolated cardiomyocytes. Diastolic Ca\textsuperscript{2+} levels were measured using indo-1 and conditions described in Figures 1 and 2. *P<0.05 vs. WT.