Genetic ablation of ryanodine receptor 2 phosphorylation at Ser-2808 aggravates Ca\(^{2+}\)-dependent cardiomyopathy by exacerbating diastolic Ca\(^{2+}\) release

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Key points

- Phosphorylation at Ser-2808 is suggested to result in RyR2 hyperactivity, i.e. ‘leakiness’, thus contributing to the pathology of cardiac diseases.
- We studied the effect of disabling phosphorylation at Ser-2808 of RyR2 in a genetic model of Ca\(^{2+}\)-dependent cardiomyopathy, which was caused by leaky RyR2.
- RyR2 phosphorylation was high at Ser-2808 in myocytes expressing wild-type (WT) RyR2; protein phosphatase increased RyR2 leakiness in cells expressing WT, but not in mutant RyR2s with disabled Ser-2808 phosphorylation sites.
- Rather than alleviating cardiac disease, ablation of the Ser-2808 exacerbated the disease phenotype by reducing survival, impairing \textit{in vivo} cardiac function and enhancing RyR2 Ca\(^{2+}\) leak and mitochondrial damage.
- These results suggest a novel mode of RyR2 regulation via dephosphorylation at Ser-2808 in normal and diseased hearts.

Abstract Phosphorylation of the cardiac ryanodine receptor (RyR2) by protein kinase A (PKA) at Ser-2808 is suggested to mediate the physiological ‘fight or flight’ response and contribute to heart failure by rendering the sarcoplasmic reticulum (SR) leaky for Ca\(^{2+}\). In the present study, we examined the potential role of RyR2 phosphorylation at Ser-2808 in the progression of Ca\(^{2+}\)-dependent cardiomyopathy (CCM) by using mice genetically modified to feature elevated SR Ca\(^{2+}\) leak while expressing RyR2s that cannot be phosphorylated at this site (S2808A). Surprisingly, rather than alleviating the disease phenotype, constitutive dephosphorylation of Ser-2808 aggravated CCM as manifested by shortened survival, deteriorated \textit{in vivo} cardiac function, exacerbated SR Ca\(^{2+}\) leak and mitochondrial injury. Notably, the deteriorations of cardiac function, myocyte Ca\(^{2+}\) handling, and mitochondria integrity were consistently worse in mice with heterozygous ablation of Ser-2808 than in mice with complete ablation. Wild-type (WT) and CCM myocytes expressing unmutated RyR2s exhibited a high level of baseline phosphorylation at Ser-2808. Exposure of these CCM cells to protein phosphatase 1 caused a transitory increase in Ca\(^{2+}\) leak attributable to partial dephosphorylation of RyR2 tetramers at Ser-2808 from more fully phosphorylated state. Thus, exacerbated Ca\(^{2+}\) leak through partially dephosphorylated RyR2s accounts for the prevalence of the disease phenotype in the heterozygous S2808A CCM mice. These results do not support the importance of RyR2 hyperphosphorylation in Ca\(^{2+}\)-dependent heart disease, and rather suggest roles for the opposite process, the RyR2 dephosphorylation at this residue in physiological and pathophysiological Ca\(^{2+}\) signalling.
Introduction

Abnormal calcium (Ca\(^{2+}\)) release from the sarcoplasmic reticulum (SR) is recognized to play a key role in pathological events in the heart. Evidence from human and animal models of disease suggests that the cardiac SR Ca\(^{2+}\) release channel (RyR2) becomes abnormally active, i.e. ‘leaky’, in the failing heart (Marx et al. 2000; Shannon et al. 2003; Ei
er et al. 2004; Kubalova et al. 2005). Accelerated diastolic release is thought to contribute to heart failure by compromising systolic Ca\(^{2+}\) release and contractility, impairing diastolic relaxation, as well as by activating pathological hypertrophic pathways and inducing cell death (Molkentin, 2000; Backs & Olson, 2006; Bers et al. 2006; Heineke & Molkentin, 2006; Anderson et al. 2011).

Although the pathological role of increased diastolic release via leaky RyR2s in heart failure has been well established, the mechanisms underlying RyR2 dysfunction remain controversial. Marks and colleagues have put forth evidence that increased phosphorylation of the RyR2 homotetramer by protein kinase A (PKA) on Ser-2808 results in dissociation of FKBP12.6 from the RyR2 complex, causing RyR2s to become hyperactive (Marx et al. 2000; Wehrens et al. 2006; Shan et al. 2010). However, key facets of this hypothesis have been challenged by other groups (Xiao et al. 2004; Carter et al. 2006; Benkusky et al. 2007; Bers, 2012; Zhang et al. 2012; Fischer et al. 2013). A growing body of evidence suggests that rather than PKA phosphorylation at Ser-2808, phosphorylation by CaMKII at Ser-2814 contributes to HF by enhancing RyR2 leak (Belevych et al. 2011; Respress et al. 2012; Fischer et al. 2013). Further adding to the complexity of the issue, RyR2 phosphorylation at these different sites have been suggested to play different roles in ischaemic vs. non-ischaemic cardiac disease (e.g. Ser-2808 vs. Ser-2814, respectively; Wehrens et al. 2006; Respress et al. 2012).

Unlike other PKA phosphorylation target proteins thought to be involved in the \(\beta\)-adrenergic modulation of cardiac Ca\(^{2+}\) signalling, such as Ser-2030 of RyR2 and Ser-16 of phospholamban (PLB; Xiao et al. 2005; Benkusky et al. 2007), Ser-2808 is highly phosphorylated (\(>60–80\%\)) even at baseline (i.e. absence of adrenergic stimulation), according to most reports (Carter et al. 2006; Xiao et al. 2006; Benkusky et al. 2007; Fischer et al. 2013). This would substantially limit the functional responsiveness of this site to PKA phosphorylation. Indeed, a number of studies reported no discernible effects of PKA stimulation on RyR2 Ca\(^{2+}\) release (Li et al. 2002; Stange et al. 2003; Benkusky et al. 2007). Interestingly, previous work by this group and others has shown that decreasing (rather than increasing) RyR2 phosphorylation at this site by exogenous phosphatases results in increased RyR2 activity and enhanced diastolic SR Ca\(^{2+}\) release (Lokuta et al. 1995; Valdivia et al. 1995; Terentyev et al. 2003). These controversial results further suggest that the role of RyR2 Ser-2808 phosphorylation is more complex than currently recognized. Thus, whether and how PKA phosphorylation status of RyR2 affects RyR2 function and contributes to degenerative cardiac disease requires further investigation.

Heart failure is associated with elevated plasma catecholamines and increased \(\beta\)-adrenergic tone as part of the compensatory response to decreased contractility (Packer, 1988). Given the complex interplay between Ca\(^{2+}\) homeostasis, \(\beta\)-adrenergic signalling, and heart failure, it is difficult to dissect the specific contribution of either pathway to the pathophysiology of this disease. To examine the relationship between Ca\(^{2+}\) leakage and cardiac disease, we recently generated a mouse model that reproduces constitutive elevated diastolic Ca\(^{2+}\) release by combining deficiency in the expression of the RyR2 regulatory protein calsequestrin 2 (CASQ2) with over-expression of SERCA1a. The double mutant (DM) mice develop dilated cardiomyopathy and die prematurely of contractile failure due to mitochondrial Ca\(^{2+}\) damage and apoptosis, with severity of the phenotype dependent on the extent of leakiness (Kalyanasundaram et al. 2012).

In the present study, we sought to examine the potential role of RyR2 phosphorylation at Ser-2808 in the development of Ca\(^{2+}\)-dependent cardiomyopathy (CCM) by using triple mutant (TM) mice featuring elevated diastolic release while expressing RyR2s that cannot be phosphorylated at this site (TM-S2808A).
If phosphorylation of Ser-2808 indeed contributes to disease by exacerbating SR Ca\(^{2+}\) leak, ablation of this site would alleviate CCM. Rather than alleviating the disease phenotype, constitutive dephosphorylation of Ser-2808 markedly aggravated CCM. These results suggest a novel mode of regulation of RyR2, in which dephosphorylation from a highly phosphorylated state (rather than further phosphorylation) increases RyR2 activity.

**Methods**

**Ethical approval**

All animal procedures were approved by The Ohio State University Institutional Animal Care and Use Committee. The study conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). The authors have read, and the experiments comply with, the policies and regulations of *The Journal of Physiology* given by Drummond (1996).

**Generation of triple mutant mouse models**

Previously, a double mutant mouse model of Ca\(^{2+}\)-dependent cardiomyopathy was generated by cross-breeding the CASQ2 knockout (KO) mice with mice overexpressing SERCA1a (Kalyanasundaram et al. 2012). The double mutant mice (CASQ2\(^{-/-}\)–SERCA1a\(^{ox}\)) of mixed strain on a predominantly FVB/N background, were further crossbred with RyR2 S2808A mice (Sv129) (Benkusky et al. 2007; Ullrich et al. 2012) to obtain triple mutant mice. The genotypes of the crossbred mice were confirmed by polymerase chain reactions (PCR; for CASQ2 KO, SERCA1a overexpression, RyR2 S2808A mutation) using tail DNA. The detailed breeding scheme is shown in Fig. 1. Age- and litter- controlled mice were utilized for experiments. Since survival of DM mice was similar between males and females, animals of both genders were used in the study. Of note, the sex and strain backgrounds of the animals differ from our previous work (Kalyanasundaram et al. 2012), where only males were used (DM females exhibited longer survival, unpublished observation). To simplify the nomenclature of the genetically engineered mice involved in this study, the term mutant (as in double mutant, DM, or triple mutant, TM) is used as a generic term to include both transgenic and targeted mutant (knock-in and knock-out) mice.

**Transthoracic echocardiography**

Mice were lightly anaesthetized using 1% isoflurane in 95% oxygen. The body temperature of the mice was maintained at \(\sim 37^\circ\text{C}\) by a heating pad. *In vivo* cardiac function was assessed at different time points using a Visualsonic Vevo 2100 imaging system, with a 30 MHz probe (VisualSonics, Toronto, Canada). Data was analysed using VisualSonic Software.

**Electrocardiographic Recordings**

Mice were lightly anaesthetized using 1.5% isoflurane in 95% oxygen. Baseline ECG was recorded for 5 min, followed by an additional 25 min after administration of \(\beta\)-agonist isoproterenol (ISO, 1.5 mg kg\(^{-1}\), i.p. injection). Response to toe-pinch reflex was examined to ensure the proper level of anaesthesia.

**Cardiomyocyte isolation and confocal Ca\(^{2+}\) imaging**

**Myocyte isolation.** Mouse ventricular myocytes were isolated as previously described (Gyorke et al. 1997). Briefly, hearts were quickly excised and perfused on a Langendorff’s apparatus at 37°C. After \(\sim 5\) min perfusion with nominally Ca\(^{2+}\)-free Tyrode solution (containing, in mM: 140 NaCl, 5.4 KCl 0.5 MgCl\(_2\), 10 Hepes and 5.6 glucose; pH 7.3), the perfusate was then switched to Tyrode solution containing Liberase Blendzymes (Roche, Applied Science, IN, USA) for digestion of the connective tissue. After \(\sim 20\) min digestion, single ventricular myocytes were isolated from dissected and triturated ventricles and stabilized in BSA containing Tyrode solution.

**Ca\(^{2+}\) imaging.** Ventricular myocytes were loaded with \(\sim 8\ \mu\text{M} \) Fluo 3 AM (Invitrogen, Carlsbad, CA, USA) for 25 min at room temperature (RT), followed by 25 min incubation at RT to allow the fluorescent dye to wash out, and deesterification. Fluo 3 was excited with the 488 nm line of an argon laser and emission was collected at 500–600 nm. Fluo 3 fluorescence was recorded in the line scan mode of the confocal microscope (Olympus Fluoview 1000). Myocytes were paced at 0.5 Hz using extracellular platinum electrodes. To assess the SR Ca\(^{2+}\) load, 20 mM caffeine was applied at the end of the experiments. Fluo 4 pento potassium salt (Invitrogen) was used to record Ca\(^{2+}\) sparks. The myocytes were permeabilized with saponin (0.01% for \(\sim 30\) s) dissolved in the internal solution, which contained (mM): 120 potassium aspartate, 20 KCl, 0.81 MgCl\(_2\), 1 KH\(_2\)PO\(_4\), 0.5 EGTA, (free [Ca\(^{2+}\)] 50 nM), 3 MgATP, 10 phosphocreatine, 20 Hepes (pH 7.2) and 5 U ml\(^{-1}\) creatine phosphokinase.

**Mitochondrial membrane potential measurement**

A voltage-sensitive fluorescent indicator, tetramethylrhodamine ethyl ester (TMRE) was utilized to measure mitochondria membrane potential. Freshly isolated mouse ventricular myocytes were loaded with TMRE (75 nM, 20 min, 37°C). TMRE was excited at 543 nm with a helium–neon laser, and the emission signals were collected at 570–650 nm. TMRE fluorescence was measured as a series of X–Y confocal images.
To quantify the level of regional mitochondria damage, the areas devoid of mitochondrial staining were automatically labelled using a custom-written MATLAB program (See Fig. 10A). The program first rotated the confocal image so that the longitudinal direction of the cell was oriented vertically. Then the horizontal edges between the stained and non-stained areas were identified using image convolution with a Sobel operator. The algorithm then paired two edges if there was no staining between them. Finally, the area between the paired edges was measured and was considered as damaged region if its area was larger than the size of 15 pixels. The individual size and number of such regions were quantified, summarized, and compared among groups.

Western blots

Mouse ventricular homogenates were prepared as previously described (Jaehnig et al. 2006). Proteins were separated by SDS–PAGE (5%–15% gradient gel, Bio-rad) and transferred onto nitrocellulose membrane. Membranes were probed by primary antibodies against RyR2 (Thermo Scientific) and Ser-2808 phosphorylated RyR2 (Badrilla Ltd), followed by secondary anti-mouse or anti-rabbit antibodies. SuperSignal chemiluminescence (Pierce Biotechnology Inc, Rockford, IL, USA) was utilized to detect the horseradish peroxidase conjugated protein bands. ImageJ was utilized to quantify the protein expression levels.


[^3]H]Ryanodine binding assay was performed as previously described with modifications (Li & Chen, 2001). The Ca^{2+}-dependent[^3]H]ryanodine binding was determined with cardiac homogenates plus a standard mixture of 200 mM KCl, 25 mM Tris–50 mM Hepes (pH 7.4), 3 mM MgATP, 1 mM EGTA, 5 mM[^3]H]ryanodine (68.4 Ci mmol−1, Dupont NEN, Wilmington, DE, USA) and CaCl2. Free Ca^{2+} concentration was set from pCa 8 to pCa 3 by using MaxChelator (http://www.stanford.edu/~cpatton/maxc.html).[^3]H]ryanodine binding was normalized to RyR2 expression level which was determined previously described (Jaehnig et al. 2006). The tissue was fixed over-night at 4°C. The tissue was further processed as previously described (Fraysse et al. 2010). Ultra-thin sections (70 nm) were produced on a Leica EM UC6 ultramicrotome and stained with 2% uranyl acetate and Reynold’s lead citrate. TEM was performed on a FEI Tecnai G2 Spirit TEM at 80 kV. Images were captured using an AMT 2 × 2 digital camera.

Materials

Protein phosphatase 1 (PP1) was purchased from Sigma (St Louis, MO, USA). Fluorescent dyes were purchased from Invitrogen (Carlsbad, CA, USA).

Statistical analysis

Results are expressed as means ± SEM. Statistical significance of differences between experimental groups was determined using unpaired Student’s t test. A value of P < 0.05 was considered statistically significant.

Results

Ablation of RyR2 phosphorylation at Ser-2808 increased mortality in CCM

To investigate the role of PKA phosphorylation of RyR2 in Ca^{2+}-dependent cardiomyopathy (CCM), we cross-bred double mutant (DM) mice deficient in CASQ2 and expressing SERCA1a with mice in which Ser-2808 of RyR2 was replaced with Ala to prevent phosphorylation at this site. The crossing gave rise to triple mutant (TM) mice that were deficient in expression of CASQ2, overexpressed SERCA1a, and expressed RyR2s that had the Ser-2808 site either partially or completely disabled (TM-S2808A+/− and TM-S2808A+/, respectively; Fig. 1). In line with our previous observations (Kalyanasundaram et al. 2012), DM mice (CASQ2+/−SERCA1a+/+) exhibited shorter survival than WT controls (albeit to a lesser extent than in the previous study). Disabling the Ser-2808 site increased the death rate in the TM mice. Surprisingly, early death was more pronounced in mice with heterozygous (TM-S2808A+/−), than with homozygous ablation of the Ser-2808 phosphorylation site (TM-S2808A+++) (Fig. 2A). Thus, the survival studies showed that genetic disabling of PKA phosphorylation at Ser-2808 did not improve survival of CCM mice. Instead, ablation of the Ser-2808 site exacerbated mortality, most notably in TM mice heterozygous for S2808A.

Of note, the crossbreeding between DM and S2808A+/+ mice gave rise to a spectrum of mice with various genotypes. All the relevant control groups for this study, including DM controls, were littermates obtained from the crossbreeding to ensure validity of comparison.

Ablation of RyR2 phosphorylation at Ser-2808 exacerbated in vivo cardiac dysfunction in CCM but did not exacerbate arrhythmia vulnerability

Consistent with previous demonstration of structural remodelling in Ca^{2+}-dependent cardiomyopathy (Kalya
Ablation of RyR2 phosphorylation at Ser-2808 exacerbated abnormal Ca$^{2+}$ handling in CCM

Confocal Ca$^{2+}$ imaging was performed in myocytes isolated from the same four mouse groups (WT, DM, TM-S2808A+/- and TM-S2808A+/+). At baseline, consistent with our previous studies (Kalyanasundaram et al. 2012), the DM myocytes displayed increased Ca$^{2+}$ transient amplitude and increased frequency of diastolic Ca$^{2+}$ waves compared to WT myocytes (Fig. 4). The frequency of Ca$^{2+}$ waves was further increased in both TM groups, with the change being more pronounced in TM-S2808A+/- myocytes (Fig. 4B). The SR Ca$^{2+}$ content was markedly increased in all three CCM groups (DM, TM-S2808A+/- and TM-S2808A+/+), with no significant differences between the groups (Fig. 4D). ISO further increased the frequency of Ca$^{2+}$ waves in each group, with both TM-S2808A+/- and TM-S2808A+/+ myocytes displaying higher wave frequencies than DM myocytes (Fig. 4B). Of note, ISO did not further increase the SR Ca$^{2+}$ content in the three CCM groups, as evidently maximum attainable SR Ca$^{2+}$ content in these cells was already reached due to the activity of overexpressed SERCA1a. The ability of ISO to increase Ca$^{2+}$ wave frequency in the TM cells expressing RyR2 that cannot be phosphorylated at Ser-2808 suggests that ISO-dependent Ca$^{2+}$ waves are mediated by phosphorylation of sites distinct from Ser-2808, most likely the CaMKII site, Ser-2814, as suggested by several previous studies (Ferrero et al. 2007, 2009; Curran et al. 2010; Pereira et al. 2013), and possibly RyR2 oxidation, which has also been shown to increase upon stimulation with ISO (Bovo et al. 2012). Additionally Chen et al. (Xiao et al. 2005, 2007) reported that PKA-dependent phosphorylation of RyR2 at Ser-2030 can increase RyR2 functional activity by modulating RyR2’s sensitivity to luminal Ca$^{2+}$ (Xiao et al. 2007). Thus the mechanisms of Ser-2808-independent effects of adrenergic stimulation remain to be clarified.

Rather than manifesting a direct modulatory influence, the observed effects of ablation of the Ser-2808 site on Ca$^{2+}$ handling in CCM mice could result from RyR2 dysregulation due to lack of CASQ2. To examine this possibility, we performed experiments in SERCA1a

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expressing mice carrying either WT or mutant RyR2 (S2808A), but normal for CASQ2. SERCA1a over-expression results in healthy mice with enhanced cardiac contractility, robust myocyte SR $\text{Ca}^{2+}$ uptake and stable SR $\text{Ca}^{2+}$ load (Loukianov et al. 1998). Similar to our results in the TM myocytes, ablation of Ser-2808 phosphorylation resulted in increased frequency of $\text{Ca}^{2+}$ sparks in SERCA1a expressing myocytes (Fig. 5). Moreover, spark frequency tended to be higher in S2808A+/− than S2808A+/+ group. Thus, ablation of Ser-2808 is capable of increasing RyR2 activity independently of CASQ2 regulation of the channel.

Ablation of Ser-2808 phosphorylation blunted the increase in $\text{Ca}^{2+}$ spark frequency by exogenous phosphatases

Consistent with previous studies (Carter et al. 2006; Xiao et al. 2006; Benkusky et al. 2007), RyR2 was highly phosphorylated (~75%) at Ser-2808 in WT and DM hearts expressing unmutated RyR2s, while substitution of Ser for Ala at 2808 rendered the sites either partially dephosphorylated or non-phosphorylatable in the TM-S2808A+/− or TM-S2808A+/+ group, respectively (Fig. 6A and B). Given the high phosphorylation status

**Figure 2. Ablation of RyR2 phosphorylation at Ser-2808 exacerbated survival and in vivo cardiac function in the CCM model**

A, survival studies on DM mice and TM mice with Ser-2808 phosphorylation ablated. Sample sizes, indicated next to the curves, included 9 males and 15 females for DM; 11 males and 14 females for TM-S2808A+/ and 11 males and 13 females for TM-S2808A+/−. B, mean ± SEM of HW/BW. C, means ± SEM of LV ejection fraction (EF%), left ventricular end-systolic dimension (LVEDD) and left ventricular end-diastolic dimension (LVEDD) at the age of 2 months. *P < 0.05 vs. WT control, #P < 0.05 vs. TM-S2808A+/−. Sample sizes are indicated within the respective columns. D, representative images of M-mode echocardiography.
of RyR2 at baseline, it is possible that dephosphorylation (rather than further phosphorylation) of this site leads to exacerbated CCM and abnormal Ca\(^{2+}\) cycling by enhancing RyR2 leakiness in TM mice. To directly examine the functional consequences of RyR2 dephosphorylation at Ser-2808, we performed experiments with application of the protein phosphatase PP1 to permeabilized DM, TM-S2808A+/- and TM-S2808A+/+ myocytes. Consistent with the notion that dephosphorylation causes increased RyR2 leakiness, baseline spark frequency was significantly higher in TM myocytes than in DM myocytes. Interestingly, baseline frequency was highest in TM-S2808A+/- myocytes with incomplete dephosphorylation (Fig. 6C and D). Addition of PP1 (10 U ml\(^{-1}\)) significantly increased spark frequency measured within 30 s after PP1 application in DM cells (Fig. 6C and D). Notably, the increase in spark frequency in these cells was transient, despite overexpression of SERCA1a which should preclude significant SR Ca\(^{2+}\) depletion in these myocytes. Since on addition of PP1, phosphorylated RyR2s must initially pass through sets of partially dephosphorylated states before becoming completely dephosphorylated at all sites, these results reaffirm that partial dephosphorylation of RyR2 at Ser-2808 exacerbates RyR2 leakiness to a greater extent than complete dephosphorylation. In further support of this notion, addition of PP1 failed to produce a transient increase of spark frequency in TM-S2808A+/- cells from

![Figure 3](image.png)

**Figure 3.** Ablation of RyR2 phosphorylation at Ser-2808 did not further increase the arrhythmogenic propensity in the Ca\(^{2+}\)-dependent cardiomyopathy models

A, the number of mice displaying ventricular tachycardia (VT) was similar among all three cardiomyopathy groups after catecholamine challenge (ISO, 1.5 mg kg\(^{-1}\) i.p.). B, representative traces of normal ECG (upper trace) and ventricular tachycardia (lower trace).
Figure 4. Ablation of RyR2 phosphorylation at Ser-2808 in the CCM model led to increased propensity for Ca²⁺ waves in isolated myocytes

A, representative line-scan images (top) and time-dependent profiles (bottom) of spontaneous Ca²⁺ waves (SCWs) under baseline condition and in the presence of 100 nmol ISO. B, pooled data for frequency of SCWs. C, pooled data for amplitude of Ca²⁺ transients, baseline and in 100 nmol ISO. #P < 0.05 vs. TM-S2808A+/−. D, pooled data for amplitude of caffeine induced Ca²⁺ transients, baseline and 100 nmol ISO.
its elevated baseline level, but instead led to significant decrease in steady-state Ca\textsuperscript{2+} spark frequency. PP1 failed to increase Ca\textsuperscript{2+} spark frequency in TM-S2808A+/+- myocytes, in which all 2808 sites are constitutively dephosphorylated (Fig. 6C and D). Taken together these results suggest that (1) RyR2 dephosphorylation at Ser-2808 enhances RyR2 leakiness and (2) the effects of partial dephosphorylation on leak are greater than those of complete dephosphorylation.

To further examine the effects of ablation of Ser-2808 phosphorylation on RyR2 activity in our CCM mouse models, we performed measurements of \[^{3}H\]ryanodine binding. Consistent with spark measurements, \[^{3}H\]ryanodine binding was significantly increased in both TM groups, and more substantially in TM-S2808A/+, as compared with DM (Fig. 7).

**Heterozygous ablation of RyR2 phosphorylation at Ser-2808 increased Ca\textsuperscript{2+} signalling heterogeneity**

Apart from increasing leakiness of individual RyR2s, it is also possible that partial disruption of RyR2 phosphorylation by PKA plays a detrimental role by increasing spatial heterogeneity of Ca\textsuperscript{2+} signalling in cardiac myocytes. Assuming that WT and mutant (S2808A) RyR2 monomers randomly mix to assemble into a tetrameric channel, one would expect a substantial heterogeneity in the composition of individual RyR2 tetramers, as well as in the composition of clusters of RyR2 channels forming release sites. To look for such possible spatial heterogeneities of Ca\textsuperscript{2+} signalling, we examined Ca\textsuperscript{2+} spark amplitude characteristics in permeabilized myocytes from the two triple transgenic groups. Notably, spark amplitude was significantly increased in TM-S2808A+/− myocytes compared to TM-S2808A++/+ (Fig. 8A and C). The difference in spark amplitudes is further emphasized in Figs 8A and B which show that the increase in Ca\textsuperscript{2+} spark amplitude was due to the redistribution of Ca\textsuperscript{2+} sparks to a larger amplitude population. Additionally, the mean standard deviation (SD) of Ca\textsuperscript{2+} spark amplitude in the S2808A+/− group was significantly larger than that of the S2808A++/+ group (Fig. 8C), further suggestive of greater spark amplitude heterogeneity in S2808A+/− myocytes.

**Ablation of RyR2 phosphorylation at Ser-2808 exacerbated ultrastructural and regional mitochondrial damage**

Elevated diastolic SR Ca\textsuperscript{2+} release has been shown to result in mitochondrial Ca\textsuperscript{2+} overload and injury, in turn leading to myocyte degeneration and death (Dorn, 2013). To examine whether deteriorated Ca\textsuperscript{2+} cycling in the TM myocytes is associated with increased ultrastructural and regional mitochondrial damage, we utilized electron microscopy (EM) and performed mitochondrial membrane potential measurements using tetrakis(ethylrhodamine ethyl ester) (TMRE). As illustrated by the EM micrographs in Fig. 9, compared with the intact mitochondria structure of WT mice, DM did not show pronounced mitochondria damage, but both TM groups had severe mitochondria damage marked by partial or complete disruption of the mitochondria matrix. The extent of such mitochondria damage was more severe in the TM-S2808A+/− group than the TM-S2808A++/+ group (Fig. 9A and B).

Mitochondrial membrane potential measurements revealed further pathological changes in mitochondria associated with deteriorated Ca\textsuperscript{2+} handling in the TM myocytes. As shown in Fig. 10, WT myocytes displayed a relatively homogenous labelling, with organized arrangement of mitochondria bands along the myofibrils. However, such homogenous labelling was disrupted to different degrees in the three CCM groups, which is illustrated more clearly in the enlarged images shown in Fig. 10A. Myocytes from the three CCM groups displayed round areas of varying sizes devoid of mitochondrial potential staining indicative of regional damage (Fig. 10A). In TM-S2808A+/− myocytes, this ‘Swiss cheese’ pattern was most pronounced. When quantified, the average size...
Figure 6. Ablation of Ser-2808 phosphorylation blunted the modulatory effect of phosphatase to increase RyR2 activity.

A. Western blots of WT hearts perfused with or without ISO (100 nmol; \( n = 3 \)).

B. Western blots of cardiac tissue homogenates in WT and the three CCM groups, *\( P < 0.05 \) vs WT (\( n = 4 \)).

C. Representative line-scan images of \( \text{Ca}^{2+} \) sparks in permeabilized myocytes before and after exposure of cells to 10 U ml\(^{-1}\) PP1. 

D. Averaged spark frequency under different experimental conditions. Data are means ± SEM from at least 8 cells from each group. *\( P < 0.05 \) vs basal. #\( P < 0.05 \) vs TM-S2808A+/−.
of regional damage (shown as holes in the TMRE staining of myocytes, highlighted in green in the lower panels of Fig. 10A) was significantly larger in the TM-S2808A+/- group than in the other two groups. When the distribution of hole size was plotted, the TM-S2808A+/- group also outnumbered the other two groups at the large-size scale (inset of Fig. 10B). Additionally, the size of mitochondria regional damage is also more heterogeneous in the TM-S2808A+/- group (Fig. 10C), supported by the increased SD of mitochondria damage size.

**Discussion**

Despite significant effort, the role of RyR2 phosphorylation by PKA in the ‘fight or flight’ response and pathophysiology of heart failure remains one of the most noted controversies in the field. Marks and coworkers have provided evidence in support of such a role via phosphorylation on Ser-2808 increasing RyR2 activity (Marx et al. 2000; Wehrens et al. 2006; Shan et al. 2010). Yet others found no indications that RyR2 PKA phosphorylation perceptibly affected Ca\(^{2+}\) handling and contractility in normal and diseased hearts (Li et al. 2002; Xiao et al. 2005; Benkusky et al. 2007; Respress et al. 2012; Zhang et al. 2012); some studies even reported that RyR2 dephosphorylation (rather than phosphorylation) at this site increased RyR2 activity (Valdivia et al. 1995; Terentyev et al. 2003). This long lasting controversy is a likely indication that RyR2 regulation is more complex than currently realized, thus resulting in a range of apparently inconsistent results. In the present study, we used a mouse model of Ca\(^{2+}\)-dependent cardiomyopathy to examine the consequences of preventing RyR2 phosphorylation at Ser-2808 on progression of Ca\(^{2+}\)-dependent disease. In this model, reduced expression of the RyR2 stabilizing protein, CASQ2, is combined with overexpression of SERCA1a to generate sustained diastolic Ca\(^{2+}\) release. These mice died prematurely, and displayed cardiac dysfunction and dilated hypertrophy associated with enhanced diastolic Ca\(^{2+}\) release, Ca\(^{2+}\)-dependent mitochondrial damage, and myocyte death in a manner proportional to the extent of diastolic release (Kalyanasundaram et al. 2012). In the present study, using this model we found that inhibition of RyR2 phosphorylation at Ser-2808, instead of alleviating, exacerbated Ca\(^{2+}\) mishandling and cardiac dysfunction. Notably, ablation of this site acted in a heterozygous disadvantage manner, that is, the consequences of partial, heterozygous ablation were more severe than those of complete, homozygous ablation. These results do not support the importance of RyR2 hyperphosphorylation in Ca\(^{2+}\)-dependent heart disease, and rather suggest roles for the opposite process, the RyR2 dephosphorylation at

![Image](https://example.com/image1.png)

**Figure 7. Ablation of RyR2 phosphorylation at Ser-2808 increased Ca\(^{2+}\) dependence of \[^{3}H\]ryanodine binding [\[^{3}H\]ryanodine binding in DM, TM-S2808A+/- and TM-S2808A+/- at different Ca\(^{2+}\) concentrations normalized to total RyR2. *P < 0.05 vs. DM.**
Consistent with previous studies (Carter et al. 2006; Xiao et al. 2006; Benkusky et al. 2007), Ser-2808 phosphorylation was relatively high (75%) at baseline condition in both WT and DM mice, even in the absence of β-adrenergic stimulation (Fig. 6A). This is in sharp contrast with the phosphorylation status of other sites (e.g. Ser-2030 of RyR2, Ser-16 of PLB) that are low at baseline and increase upon β-adrenergic stimulation (Xiao et al. 2005; Benkusky et al. 2007). Therefore, to account for the deterioration of the CCM phenotype in TM mice, and in particular of those heterozygous for S2808A, we hypothesized that dephosphorylation from the more fully phosphorylated states at Ser-2808 makes the RyR2 channels leakier, thereby exacerbating CCM. In support of this hypothesis, application of PP1 produced a surge in spark frequency in DM myocytes expressing WT RyR2 highly phosphorylated at baseline, which was absent in TM myocytes expressing RyR2s with fully or partially disabled Ser-2808 phosphorylation sites (Fig. 6). Interestingly, the increase in sparks in DM myocytes was transient, consistent with a transition of RyR2s from highly phosphorylated states to only partially dephosphorylated, but functionally more active states, and then to completely dephosphorylated states with less pronounced leakiness (Fig. 6). In further support of this explanation, the baseline spark frequency for TM myocytes was significantly higher than that of DM myocytes, with the difference being most pronounced in the TM-S2808A+/− myocytes that mimic partially dephosphorylated states (Fig. 6). Moreover, [3H]ryanodine binding was significantly increased in TM hearts, especially in the TM-S2808A+/− group, which is indicative of increased RyR2 activity in these groups (Fig. 7). These results are also supported by previous reports showing that RyR2 dephosphorylation can increase RyR2 activity in various experimental settings (Lokuta et al. 1995; Valdivia et al. 1995; Terentyev et al. 2003). The heterozygous-disadvantaged manner in which genetic modification affected the RyR2 tetramer is not unique to this channel. Similar effects have been reported for a mutation in the HCN4 channel (Duhme et al. 2014), which is also a tetramer. Apparently, partial modification of tetramers alters monomer interaction, thus affecting channel stability and functional activity (Duhme et al. 2014).

Notably, in addition to increased overall RyR2 leakiness and mitochondrial damage, TM myocytes, particularly those heterozygous for the S2808A modification, exhibited profound heterogeneities in Ca$$^{2+}$$ signalling and regional mitochondrial degradation (‘Swiss cheese’

**Mechanisms of action of constitutive dephosphorylation of Ser-2808 in CCM**

this residue in physiological and pathophysiological Ca$$^{2+}$$ signalling.

**Figure 9. Heterozygous ablation of RyR2 phosphorylation at Ser-2808 in the CCM model led to more severe ultrastructural remodelling than homozygous ablation and unmodified Ser-2808**

Figure 10. Mitochondrial membrane potential measurements revealed severe regional damage in TM-S2808A+/−.

A, representative images of mitochondrial membrane potential measurements, the top panel shows the overview of TMRE stained cardiac myocytes, enlarged regions of the boxed areas are shown in the lower left corner, with regional damage or holes highlighted by a MATLAB program in green. B, the histograms of distribution of mitochondria regional damage size. C, quantification of regional damage or holes: the average number of holes (normalized to the size of myocyte), the average size of holes (normalized to the size of myocyte) and the average standard deviation (SD) of hole size. #P < 0.05 vs. TM-S2808A+/−.
pattern). These results suggest that the impact of increased leakiness of dephosphorylated RyR2s in TM-S2808A+/- myocytes is further amplified by heterogeneity of Ca\(^{2+}\) handling expected as a result of combinatorial variations in the monomer composition of individual RyR2 tetramers. Indeed, the RyR2 channel is a protein tetramer assembled from the available monomer variants (Nakai et al. 1990; Fill & Copello, 2002); thus, in myocytes expressing the two monomer variants, most channels will be composed of a mixture of the phosphorylated WT and non-phosphorylatable mutant monomers, with a smaller fraction of channels composed of the WT or mutant monomers only (i.e. 87.5%, 6.25% and 6.25%, respectively; Xu et al. 2008; Loaiza et al. 2012). Our results are consistent with other examples of degenerative processes and localized Ca\(^{2+}\)-dependent mitochondrial damage caused by disruption in homogeneity of RyR2 function in both cardiac and skeletal muscle (Litwin et al. 2000; Diaz et al. 2002; Aistrup et al. 2006; Zhou et al. 2010).

When recently examining Ca\(^{2+}\) handling and RyR2 function in WT vs. S2808A mice, Ullrich et al. (2012) found only subtle differences. However, these studies were performed in mice homozygous for S2808A; furthermore, most experiments were conducted in the presence of ISO, which is expected to lead to RyR2 modifications through other mechanisms, including CaMKII phosphorylation at Ser-2814 and oxidation (Ferrero et al. 2007; Curran et al. 2010; Bovo et al. 2012), thus masking potential effects specifically related to the phosphorylation status of Ser-2808. Therefore, future experiments are needed to determine as to what extent this phenomenon applies to other disease conditions and physiological settings.

Lastly, it is to be noted that survival of DM mice in this study is substantially longer than what we have previously reported in mice bearing the same genetically-engineered modifications (Kalyanasundaram et al. 2012). This difference could be ascribed to the differences in the genetic background and gender of the animals used as specified in the Methods. Genetic background and gender differences may also contribute, in part, to the multiplicity of findings in the literature and their role requires further investigation.

**Implications for normal physiology and disease**

Our study demonstrates an unexpected functional complexity of RyR2 modification through phosphorylation/dephosphorylation. Cardiac performance and Ca\(^{2+}\) handling are under balanced control of sympathetic and parasympathetic systems. Upon sympathetic stimulation, phosphorylation of RyR2 at Ser-2814 and other target proteins including L-type Ca\(^{2+}\) channel and PLB contributes to the ‘fight or flight’ response by facilitating Ca\(^{2+}\) cycling (De Jongh et al. 1996; Chu et al. 2000; Pereira et al. 2013). In contrast, Ser-2808 phosphorylation does not seem to play a major role in this process. This is supported by our results showing that ISO can facilitate Ca\(^{2+}\) cycling independent of the S2808A mutation (Fig. 4).

Our investigation shed light on a novel mode of RyR2 operation, possibly evolved to mediate SR Ca\(^{2+}\) release at low levels of cardiac energy consumption upon shifting the regulatory balance to the parasympathetic side. The dependence of Ca\(^{2+}\) release on SR Ca\(^{2+}\) content is highly non-linear such that lowering SR Ca\(^{2+}\) content below a level as high as 40% of normal can abolish release (Bassani et al. 1995). Thus, increasing RyR2 activity through dephosphorylation at Ser-2808, as shown here, coupled with dephosphorylation of PLB and L-type Ca\(^{2+}\) channel aimed at reducing the SR Ca\(^{2+}\) load could maximize energy efficiency of Ca\(^{2+}\) cycling. In support of this notion, ablation of the Ser-2808 phosphorylation sites increased Ca\(^{2+}\) spark frequency not only in CCM myocytes but also in myocytes from healthy SERCA1a over-expression mice (Figs 5 and 6). Previously, it has been suggested that reducing RyR2 phosphorylation at Ser-2808 alleviates heart failure by decreasing SR Ca\(^{2+}\) leak. Our results obtained in a genetic model of Ca\(^{2+}\)-dependent cardiomyopathy suggest that the relationship between RyR2 phosphorylation/dephosphorylation at this site and cardiac disease is much more complex and warrants further investigation in clinically more relevant models.

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Additional information

Competing interests

There are no competing interests.

Author contributions

B.L. and S.G. contributed to the conception, design, data interpretation and manuscript preparation. B.L., H.-T.H., F.V. and C.V. contributed to data collection and analysis. All authors approved the final version of the manuscript.

Funding

This work was supported by the National Institutes of Health (RO1 HL088635 to B.C.K.; RO1 HL074045 and HL063043 to S.G.) and American Heart Association (postdoctoral fellowship 13POST16910102 to B.L.).

Acknowledgements

We thank Drs Jonathan Davis and Xander Wehrens for critical reading of the manuscript.
Translational perspective

The cardiac ryanodine receptor (RyR2) is the Ca\(^{2+}\) release channel of sarcoplasmic reticulum that provides the Ca\(^{2+}\) necessary to induce cardiac contraction. Excessive RyR2 activity during diastole (i.e. RyR2 Ca\(^{2+}\) leak) is thought to lead to a spectrum of cardiac pathologies (i.e. 'ryanopathies') including heart failure (HF) by compromising systolic Ca\(^{2+}\) release and contractility, impairing diastolic relaxation, and activating pathological hypertrophic pathways that trigger cell death. Post-translational modifications of RyR2, especially phosphorylation, are considered among the most important factors contributing to pathological RyR2 leakiness. However, the role of RyR2 phosphorylation in cardiac pathology remains incompletely understood and highly controversial. Previous work by some investigators has suggested that in HF, 'hyperphosphorylation' of RyR2 by PKA at Ser-2808 renders RyR2 leaky, and that genetic ablation of Ser-2808 prevents RyR2 leakiness and HF. However, these findings have not been reproduced by several other studies conducted in similar disease settings. In our current study, we utilized a genetic model of cardiomyopathy induced by excessive RyR2 Ca\(^{2+}\) leak. Remarkably, disabling Ser-2808 phosphorylation exacerbated rather than alleviated Ca\(^{2+}\)-dependent cardiomyopathy. Additionally, our results demonstrated that dephosphorylation, rather than phosphorylation of Ser-2808 – a site that is already highly phosphorylated at baseline – made RyR2 leakier and exacerbated the cardiomyopathy. Our results suggest that RyR2 modulation by Ser-2808 phosphorylation is more complex than previously thought, thereby suggesting the need for more tailored approaches in the design of HF therapies.