Hyperphosphorylation of the Cardiac Ryanodine Receptor at Serine 2808 Is Not Involved in Cardiac Dysfunction After Myocardial Infarction

Hongyu Zhang,* Catherine Makarewich,* Hajime Kubo,* Wei Wang, Jason Duran, Ying Li, Remus Berretta, Walter J. Koch, Xiongwen Chen, Erhe Gao, Hector Valdivia, Steven R. Houser

Rationale: Abnormal behavior of the cardiac ryanodine receptor (RyR2) has been linked to cardiac arrhythmias and heart failure (HF) after myocardial infarction (MI). It has been proposed that protein kinase A (PKA) hyperphosphorylation of the RyR2 at a single residue, Ser-2808, is a critical mediator of RyR dysfunction, depressed cardiac performance, and HF after MI.

Objectives: We used a mouse model (RyRS2808A) in which PKA hyperphosphorylation of the RyR2 at Ser-2808 is prevented to determine whether loss of PKA phosphorylation at this site averts post MI cardiac pump dysfunction.

Methods and Results: MI was induced in wild-type (WT) and S2808A mice. Myocyte and cardiac function were compared in WT and S2808A animals before and after MI. The effects of the PKA activator Isoproterenol (Iso) on L-type Ca2+ current (I\(_{Ca_L}\)), contractions, and [Ca2+]i transients were also measured. Both WT and S2808A mice had depressed pump function after MI, and there were no differences between groups. MI size was also identical in both groups. L type Ca2+ current, contractions, Ca2+ transients, and SR Ca2+ load were also not significantly different in WT versus S2808A myocytes either before or after MI. Iso effects on Ca2+ current, contraction, Ca2+ transients, and SR Ca2+ load were identical in WT and S2808A myocytes before and after MI at both low and high concentrations.

Conclusions: These results strongly support the idea that PKA phosphorylation of RyR-S2808 is irrelevant to the development of cardiac dysfunction after MI, at least in the mice used in this study. (Circ Res. 2012;110:00-00.)

Key Words: myocardial infarction ■ ryanodine receptor ■ heart failure ■ PKA hyperphosphorylation
more LTCCs open, to raise local junctional cleft [Ca\(^{2+}\)], to promote binding to RyRs, to cause their opening. The resultant flux of SR Ca\(^{2+}\) into the junctional space further elevates cleft Ca\(^{2+}\), and this activates other nearby RyRs to induce locally regenerative Ca\(^{2+}\) release.\(^{12}\) In normal hearts, all couplons within a cell appear to be activated with each heart beat,\(^{11}\) and it is the sum of the Ca\(^{2+}\) release from all couplons that produces the systolic Ca\(^{2+}\) transient that activates the contractile apparatus.\(^{13}\) Relaxation occurs through Ca\(^{2+}\) reuptake into the SR through the SR Ca\(^{2+}\)-ATPase (SERCa) and Ca\(^{2+}\)-extrusion through the Na/Ca exchanger.\(^{14,15}\)

Length-independent changes in the force of cardiac contraction (contractility) are critical to normal cardiac function and are largely brought about by altering the amplitude and duration of the systolic [Ca\(^{2+}\)] transient. Contractility is efficiently regulated in the normal heart and this primarily results from activation of adrenergic signaling pathways through protein kinase A (PKA). Ca\(^{2+}\) regulatory PKA targets include the LTCC (to increase Ca\(^{2+}\) current and SR Ca\(^{2+}\) load), phospholamban (to increase the activity of SERCa, promote more rapid removal of Ca\(^{2+}\) from the cytoplasm and to help increase SR Ca\(^{2+}\) loading),\(^{16}\) and RyR (to increase Ca\(^{2+}\) dependent opening).\(^{16,17}\) The roles of PKA-mediated LTCC and SERCa regulation in the normal control of cardiac contractility are widely accepted.\(^{18,19}\) However, the independent role of PKA-mediated regulation of RyR2 activity as a major regulator of cardiac contractile strength is not broadly supported in the literature.\(^{20}\) This effect is predicted from the fact that increasing the Ca\(^{2+}\) dependent opening probability of RyR2 should not increase the number of couplons that release stored Ca\(^{2+}\), since all couplons participate in normal EC coupling.\(^{21-23}\)

In cardiac disease, the contractile demands on the heart are persistently increased, and chronic activity of the sympathetic nervous system is needed to maintain cardiac function.\(^{24}\) This heightened hyperadrenergic state of the stressed heart is thought to contribute to depression of contractility reserve and cardiac arrhythmias.\(^{25}\) Many studies have shown that deranged Ca\(^{2+}\) handling in heart failure involves altered SERCa activity and reduced LTCC abundance with increased activity.\(^{6,26-27}\) The Marks laboratory has proposed that abnormal PKA-mediated phosphorylation of RyR2 (at Serine 2808) is centrally involved in defective Ca\(^{2+}\) handling in HF.\(^{9,28}\) PKA-mediated hyperphosphorylation of RyR at S2808 is proposed to increase RyR Ca\(^{2+}\)-dependent opening (leak), leading to depletion of SR Ca\(^{2+}\) stores.\(^{9}\) Studies by the Marks group have shown that PKA-mediated hyperphosphorylation of RyR2 at serine-2808 is a critical mediator of normal cardiac contractility regulation\(^{29,30}\) and cardiac dysfunction after MI.\(^{6}\) These ideas were explored in experiments with genetically modified mice in which Ser-2808 is mutated to alanine (S2808A), to eliminate PKA-mediated phosphorylation.\(^{29}\) In previous studies, we\(^{31}\) and others\(^{32}\) were unable to show any alterations in sympathetic regulation of cardiac or myocyte function in RyR S2808A mice. Given the critical nature of this topic, we studied if prevention of RyR2 phosphorylation after MI preserved cardiac function and reduced premature death, consistent with the development of cardiac dysfunction after MI being dependent on PKA phosphorylation of a single amino acid on RyR2.

In the present study, we examined if the absence of PKA phosphorylation of RyR at Ser-2808 (RyRS2808A mice) reduced the damaging effects of MI on cardiac structure and function. Cardiac performance was compared before and after MI in wild-type (WT) and RyRS2808A mice. Isoproterenol (Iso) effects on L-type Ca\(^{2+}\) current (I\(_{\text{CaL}}\)), contractions and [Ca\(^{2+}\)]\(_{\text{i}}\) transients were also measured. Our results showed that both WT and S2808A mice have depressed pump function after MI, but we could not show a difference between groups. MI size was identical in both groups. L-type Ca\(^{2+}\) current, contractions, and [Ca\(^{2+}\)]\(_{\text{i}}\) transients were also not significantly different in WT versus S2808A mice myocytes either before or after MI. Iso effects on Ca\(^{2+}\) current, contraction, and Ca\(^{2+}\) transients were identical in WT and S2808A myocytes before and after MI at both low and high concentrations. These results strongly support the idea that PKA phosphorylation of RyR-S2808 plays little or no role in the development of cardiac dysfunction after MI in these mice.

**Methods**

All methods have been described in detail in previous reports and are described in detail in the Online Supplement. Briefly, myocardial infarction was induced in WT and S2808A mice by permanent occlusion of the left anterior descending coronary artery.\(^{33}\) Cardiac function was measured with echocardiography. At the time of death, hearts were removed and used for functional studies (isovolumic hearts and isolated myocytes), tissue was used for Western or biochemical analyses, or hearts were fixed and processed for histological studies.

**Results**

**Cardiac Function In Vivo After MI**

MI was induced by permanent ligation of the left anterior descending coronary artery. Before MI, there was no significant difference in cardiac function or structure between WT and RyR-S2808A mice. Ejection fraction (EF), fractional shortening (FS), left ventricle internal diameter, and septum wall thickness were not different in WT and RyR-S2808A mice (Figure 1). These data are consistent with those we have previously reported.\(^{31}\) One week after MI, both groups of mice had significant decreases of EF (pre-MI versus 1 week
post-MI; WT: 58.14±2.81 versus 32.87±3.88; S2808A: 61.59±3.57 versus 26.90±3.36) and fractional shortening (pre-MI versus 1-week post-MI; WT: 29.08±2.72 versus 15.68±2.05; S2808A: 32.92±2.73 versus 12.13±1.90) (Figure 1A and 1B), but there was no significant difference between WT and S2808A mice. Four weeks after MI, EF and FS were still depressed and there were no differences between WT and S2808A hearts (WT versus S2808A; EF: 25.65±1.90 versus 27.14±4.07; FS: 12.82±2.08 versus 11.94±0.93). There were significant changes in ventricular geometry and wall thickness after acute MI. The LV was dilated in all hearts and chamber size was significantly increased in the first week and 4 weeks after MI (Figure 1C). Septum wall thickness was decreased significantly in the first week after MI in both groups and became stable at the end of 4 weeks (Figure 1D). There were no significant differences in geometry or wall thickness between WT and S2808A mice after MI.

Survival and Cardiac Phenotype After MI
Animals were studied for 4 weeks after MI surgery. There was no significant difference in survival between WT and S2808A mice (87% of all animals survived the 4-week study interval). Heart weight was significantly greater in MI mice compared with sham mice (Figure 2A), but there was no difference between control and S2808A hearts. Lung weight was also significantly increased after MI in both groups and became stable at the end of 4 weeks (Figure 1C). Septum wall thickness was decreased significantly in the first week after MI in both groups and became stable at the end of 4 weeks (Figure 1D). There were no significant differences in geometry or wall thickness between WT and S2808A mice after MI.

Infarct Length and Fibrosis Were Not Different in WT and S2808A Mice
Infarct length was measured 4 weeks after MI. There was no significant difference in infarct length between WT and S2808A mice (WT versus S2808A: 32.55±2.38% versus 26.55±3.03%) (Figure 2D). Representative examples of infarcted cardiac tissue and fixed heart tissue sections are shown in Online Figure I, A through C. We also measured fibrosis in noninfarcted (remote) zones of infarcted hearts. The collagen content in the remote zone was not significantly different in WT and S2808A infarcted hearts (Figure 2E). These data show that WT and S2808A mice had similar infarct size and fibrotic remodeling after MI.

Western Blot Analysis
If PKA-mediated RyR-S2808 phosphorylation plays a critical role in either the normal physiology of the mouse heart or in the progression of cardiac dysfunction after MI, then eliminating this site would be expected to induce adaptive alterations in other Ca²⁺ regulatory proteins, as shown in other studies. Ca²⁺ regulatory proteins including the L-type Ca²⁺ channel α1C subunit, phospholamban, total and phosphorylated at Thr-17 (PLBt, PT17), RyR2 total protein (Ryt), and RyR2 phosphorylated at S2808 (Ryp) were measured with Western analysis in sham and MI mice (representative blots are shown in Online Figure II). Average data are shown in Figure 3. There was no significant difference in the abundance of any of these Ca²⁺ regulatory proteins between WT and S2808A mice either in sham or MI groups (Figure 3). There was a significant increase of PLB phosphorylation at Thr17 in both WT and S2808A hearts after MI, but there were no differences between the groups. An antibody against phosphorylated RyR-Ser2808 did not react to RyR-S2808A samples. RyR Ser2808 phosphorylation was significant in sham hearts and increased after MI (Online Figure II and Figure III). These results show that there are no significant differences in Ca²⁺ regulatory protein abundance or phosphorylation either before or after MI in WT versus S2808A hearts. These results are inconsistent with a significant role for Ser2808 phosphorylation in the normal physiology of the mouse heart or in the deranged structure and function after MI.
LTCC Current \( (I_{Ca,L}) \) in S2808A Mice After MI

Elimination of a critical element in normal EC coupling or in the regulation of the contractile response to stress would be expected to have a major effect on baseline cardiac function. If not, as we have shown in this and other studies, then significant adaptation of related molecules would be needed to offset the defective or lost protein. An example would be the conditional NCX knockout mouse that survives with...
adaptive reduction in the L-type Ca\(^{2+}\) current density. If phosphorylation of RyR at Ser-2808 is critical to the regulation of EC coupling in health and disease, then its loss should either induce a significant basal phenotype (which it did not) or there should be adaptive changes in other EC coupling proteins. The major protein that would be expected to change would be the L-type Ca\(^{2+}\) channel, with a known essential role in EC coupling. Therefore, we measured ICa-L density and responsiveness to catecholamines. Peak ICa-L density was not significantly different in WT and S2808A myocytes after sham operations (WT sham: 10.71±1.78 pA/pF, n=6; S2808A sham: -11.35±2.18 pA/pF, n=5). Isoproterenol (Iso) increased ICa-L similarly in both WT and S2808A myocytes (sham WT after Iso: 16.55±0.58 pA/pF n=5; sham S2808A after Iso: 17.95±3.82 pA/pF, n=5) (Figure 4A through 4C). Iso also caused similar hyperpolarizing shifts (a signature of PKA phosphorylation effect) in the voltage dependence of ICa-L activation in sham control and S2808A myocytes (Online Figure III, A). After MI, there was also no difference in peak ICa-L in WT and S2808A myocytes (WT MI: -11.76±2.1pA/pF, n=7; S2808A MI: -9.00±0.98 pA/pF, n=7). Iso increased ICa-L significantly in both WT (-16.2±2.7 pA/pF, n=7) and S2808A (-15.2±3.0 pA/pF, n=7) myocytes, but again no significant difference was detected between groups (Figure 4A, 4B, and 4D). The voltage dependence of activation of ICa-L in S2808A myocytes was not significantly different than control myocytes either before or after Iso treatment (Online Figure III, B). Collectively, these data show that there are no significant changes in LTCC density or PKA regulation in RyR-S2808A mice either before or after MI.

Contraction and \([\text{Ca}^{2+}]_i\) Transients in WT and S2808A Mice Before and After MI

Twitch contractions and \([\text{Ca}^{2+}]_i\) transients were studied at 0.5 and 2 Hz in the absence and presence of Iso (representative data are shown in Online Figure IV and V). Low and high concentrations of Iso were tested because others have suggested that differences in catecholamine responsiveness of WT and S2808A myocytes are only observed at low concentrations.\(^{29}\) The rationale for this is unclear since the hyperphosphorylation hypothesis predicts that differences between WT and S2808A animals should be most robust when WT RyR is hyperphosphorylated. Nevertheless, we compared the effects of lower [Iso] on WT and RyR-S2808A animals and myocytes.

There were no significant differences in the amplitude of contraction (FS) or the \([\text{Ca}^{2+}]_i\) transients between WT or S2808A myocytes (0.5 and 2 Hz) either before or after MI. Low and high [Iso] caused identical changes in contraction and \([\text{Ca}^{2+}]_i\) transients in both groups under all conditions tested (Figures 5 and 6). Low [Iso] (10 nmol/L) caused robust increases in contraction and \([\text{Ca}^{2+}]_i\) transients in all myocytes, and there were no differences between WT and S2808A myocytes at any tested pacing rate or with or without MI. High [Iso] did not increase the amplitude of contraction or the \([\text{Ca}^{2+}]_i\) transient beyond that produced by 10 nmol/L Iso in either group under any conditions tested. These results
do not support the hypothesis that the loss of PKA-mediated phosphorylation of RyR2 at S2808 has any effect on baseline cardiac myocyte function in the absence or presence of low or high [Iso].

MI induced changes (half-width and tau) in the duration of contractions and [Ca\(^{2+}\)]\(_i\) transients (Figures 5 and 6 and Online Figures IV and V). These changes were identical in WT and S2808A mice. Slowing and prolongation of contraction after MI probably results from changes in a host of processes, but changes in phosphorylation of RyR2 at S2808A do not appear to be a central element of this functional remodeling.

Low and high [Iso] was also tested in vivo (Online Figure VI), and we could not define a difference in effects in WT and S2808A animals. These results are similar to those we have shown previously in isolated hearts from these animals. Collective these results suggest that PKA-mediated phosphorylation of RyR-S2808 is not critical modulator of myocyte function in health or disease, at least under our conditions.

**SR Ca\(^{2+}\) Load in WT and S2808A Myocytes**

SR Ca\(^{2+}\) load is often decreased in myocytes isolated from failing hearts, and this is thought to be, at least in part, due to an abnormal “leak” of Ca\(^{2+}\) from the SR. PKA-mediated hyperphosphorylation of RyR at S2808 has been proposed as the mechanism that leads to SR “leak” and a reduction in SR Ca\(^{2+}\) stores in hyperadrenergic states, but other studies suggest that CaMKII mediated RyR phosphorylation at an alternative site is the cause of this “leak.”

In the present experiments, SR Ca\(^{2+}\) load was measured as the caffeine releasable SR Ca\(^{2+}\) transient (Figure 7). The effects of Iso on SR Ca\(^{2+}\) load were determined in sham and MI myocytes. There were no significant differences in SR Ca\(^{2+}\) load or the decay rate of these transients (not shown) between WT and S2808A myocytes under control conditions (Figure 7C). Iso caused a significant increase in SR Ca\(^{2+}\) load in both WT and S2808A myocytes (Figure 7C), and the extent or rate of decay (not shown) was not different.

MI caused a significant reduction in SR Ca\(^{2+}\) load in both WT and S2808A myocytes, and there were no differences between groups (Figure 7C). Iso had very small effects on SR Ca\(^{2+}\) load in MI myocytes, and again there were no differences between groups (Figure 7C). These results do not support the hypothesis that hyperphosphorylation of RyR at S2808 is critical to the SR Ca\(^{2+}\) leak that develops after MI.
The present study evaluated the hypothesis that PKA-mediated phosphorylation of RyR-S2808 is critical for the normal regulation of cardiac contractility and that RyR hyperphosphorylation is critical for the cardiac dysfunction that develops after MI. Although the RyR hyperphosphorylation hypothesis has been supported by some previous studies, the topic is still controversial, and many critical issues have not been resolved.

In the normal heart, contractility is primarily regulated by the sympathetic nervous system. To increase myocyte contractility, sympathetic signaling cascades activate PKA. The PKA target proteins that become phosphorylated include the LTCC, PLB, RyR, and troponin C. Increased myocyte contractility involves an increased amplitude and reduced duration of the systolic [Ca\textsuperscript{2+}] transient. The contribution of increased Ca\textsuperscript{2+} influx through the phosphorylated LTCC complex and increased Ca\textsuperscript{2+} uptake by the SR after PLB phosphorylation are well described in many laboratories. The idea that PKA-mediated phosphorylation of RyR plays a critical role in the regulation of cardiac contractility is less well accepted and is not supported by modeling studies. Studies with a S2808A mouse from the Marks group support a significant role for RyR2 phosphorylation at S2808 in the normal regulation of cardiac contractility and in the response of the heart to pathological stress. The present results with a similar mouse are inconsistent with these previous reports.

MI reduces functional cardiac mass, and a hyperadrenergic state is required to maintain cardiac pump function. The persistent hyperadrenergic state eventually contributes to abnormal myocyte function and death that precipitates heart failure. The question posed here was if preventing phosphorylation of RyR2 at a single amino acid is sufficient to reduce adverse functional and structural remodeling after MI. Our results suggest that eliminating PKA-mediated phosphorylation at RyR-S2808 has no impact on remodeling after MI, at odds with results of studies discussed below. A number of reports strongly support the hypothesis that PKA-mediated phosphorylation of RyR at S2808 causes the RyR-associated protein FKBP12.6 (also called Calstabin) to dissociate from RyR and that this increases the Ca\textsuperscript{2+} dependent opening of RyR. Hyperphosphorylation of RyR2 by PKA has been proposed as the cause of RyR "leak" that leads to reduced SR content and contractility in HF. However, there is equally compelling data suggesting that PKA-
mediated phosphorylation of RyR at S2808 has little or no role in the regulation of myocyte function in the normal or diseased heart.31–32,38 There are studies showing that there are no appreciable differences in the level of phosphorylation of RyR2 and basal channel activity between failing and nonfailing canine hearts.38 S2808A animals with pressure overload did not have better function than WT animals.32 In addition, we have previously suggested that phosphorylation of RyR2 at Ser-2808 is not involved in the adrenergic regulation of normal cardiac contractility.31 These data are consistent with a detailed modeling study from the Bers group23 that predicts that increasing RyR Ca\(^{2+}\)/H\(^{1+}\)-mediated opening should have very little effect on contractility. In addition, others have shown that CaMKII-mediated phosphorylation of RyR at S2814 rather than PKA-mediated phosphorylation of S2808 is responsible for regulation of RyR function45 and that PKA phosphorylation of RyR does not induce significant displacement of FKBP from RyR2.46 The results we report in the present investigation are consistent with those that have been unable to document any significant role for RyRS2808 phosphorylation in health and disease.

We were unable to find any significant difference in any Ca\(^{2+}\) regulatory process in S2808A mice. Our logic for looking at other molecules involved in EC coupling and the regulation of cardiac contractility in S2808A mice was that because everyone agrees that S2808A mice have no basal phenotype, either S2808 phosphorylation is unimportant or there are adaptive changes in other proteins involved in the regulation of myocyte Ca. An example of this in mouse models is the conditional cardiac Na/Ca\(^{2+}\) exchanger (NCX) KO that was expected to die after eliminating the major Ca\(^{2+}\) efflux mechanism in the normal heart, but did not die.34 It was shown that these mice were able to live with a large reduction in Ca\(^{2+}\) influx by an adaptive reduction in Ca\(^{2+}\) influx through the L-type Ca\(^{2+}\) channel.34 If RyR-S2808 phosphorylation is a critical component of the physiological regulation of cardiac contractile function, then related adaptations would be expected because there is no basal phenotype. We were unable to detect any change in abundance, phosphorylation, or function of any Ca\(^{2+}\) regulatory protein involved in Ca\(^{2+}\) entry or efflux and SR Ca\(^{2+}\) uptake, storage, and release.

We were unable to detect any beneficial effect of loss of RyRS2808 phosphorylation after MI. Importantly we were able to show reduced SR Ca\(^{2+}\) loading in MI myocytes, but there were similar reductions in WT and S2808A myocytes. These results support the idea that alterations in SR Ca\(^{2+}\) uptake, storage, and release are involved in post MI functional remodeling but do not support a role for hyperphosphorylation of RyR S2808 in these changes.

It is difficult to find common ground with those studies that have reported such strong evidence for the RyRS2808 hyperphosphorylation hypothesis.4 A recent report49 suggested that the reason for the differences between those studies finding and not finding different catecholamine effects in WT versus

---

Figure 7. Sarcoplasmic reticulum Ca\(^{2+}\) content is identical in WT and S2808A myocytes from sham and MI hearts. Caffeine-induced SR Ca\(^{2+}\) release was measured in WT and S2808A myocytes ±Iso (10 nmol/L) from sham or MI mice. A and B. Examples of Ca\(^{2+}\) transients elicited by 0.5 Hz of electric field stimulation (first 4 traces) followed by Ca\(^{2+}\) transients induced by caffeine (fifth large trace; peak indicates SR Ca\(^{2+}\) content). C. Average amplitudes of caffeine-induced Ca\(^{2+}\) transients ±Iso in WT and S2808A myocytes. *P<0.05 between sham and MI, #P<0.05 between ±Iso.
S2808A mice is related to the fact that studies not detecting differences used Iso concentrations that were too high. Interestingly, there were already data in the literature using low [Iso] that did not support this notion. A previous study\(^{31}\) showed that exposing WT and RyR2808A isovolumic hearts to 10 nmol/L Iso caused identical increases in contractility in both groups, whereas a 10-fold higher concentration (100 nmol/L) of Iso was used in the recent studies from the Marks laboratory, reduced Iso effects were seen in S2808A hearts. Because of this concentration issue, we tested the effects of lower [Iso] on isolated myocyte function and could not find any difference in effects in WT and S2808A cells. Therefore, our present and previous results do not support the idea that low doses of catecholamines are required to demonstrate differences in cardiac function related to RyR-S2808 phosphorylation. We are at a loss to find an explanation for the differences used Iso concentrations that were too high.

In summary, our results do not support a major role of S2808 PKA phosphorylation on myocyte contractility regulation in health and disease.

**Sources of Funding**

This study was supported by grants NIH HL089312, HL033921, and HL091799 (to S.R.H.).

**Disclosures**

None.

**References**


---

**Novelty and Significance**

**What Is Known?**

- After myocardial infarction (MI), sympathetic regulatory pathways are activated (with heightened activity of protein kinase A [PKA]) to increase the contractility of surviving myocytes and thereby maintain cardiac pump performance.
- Some studies propose that PKA-mediated hyperphosphorylation of a single serine (S2808) on the ryanodine receptor (RyR); responsible for Ca2+ release from the sarcoplasmic reticulum [SR] is a critical determinant of myocyte contractile and electric dysfunction after MI.
- Other studies have suggested that phosphorylation of RyRS2808 is not an essential regulator of RyR function in health and disease.

**What New Information Does This Article Contribute?**

- Preventing phosphorylation of RyR at S2808 (with an alanine substitution; RyRS2808A) does not induce a significant change in cardiac function or in the contractile response to adrenergic agonists, at either low or high concentrations.
- MI induced similar reductions in cardiac function and myocyte adrenergic responsiveness in wild-type and RyRS2808A mice.
- Changes in Ca2+ regulatory processes after MI, including a reduction in SR Ca2+ stores, were similar in wild type and RyRS2808A mice, documenting the lack of functional benefit of preventing PKA phosphorylation of S2808 after MI.

MI causes regional cellular death in the heart and results in depressed cardiac pump function. Heightened adrenergic signaling with activation of PKA helps maintain cardiac function after MI but eventually causes myocyte dysfunction. Some investigators have suggested that PKA-mediated phosphorylation of RyR at S2808 is critical to the normal regulation of cardiac contractile function and that hyperphosphorylation of S2808 is responsible for SR disturbances that reduce contractility and promote arrhythmias in disease, making hyperphosphorylation of S2808 a therapeutic target. The results of our study show that RyRS2808 phosphorylation increases after MI and that SR Ca2+ stores are reduced, consistent with SR Ca2+ “leak” hypothesis. However, preventing RyRS2808 phosphorylation had no effect on adrenergic regulation of myocyte or heart function in the normal heart, and it did not prevent depressed function after MI or reductions in SR Ca2+ loading after MI. Our results show that, at least under the experimental conditions used, PKA-mediated phosphorylation of RyR at S2808 is not a critical regulator of cardiac contractile function in the normal heart and that it might not be the mechanism that reduces SR Ca2+ loading and myocyte contractility reserve after MI.
Hyperphosphorylation of the Cardiac Ryanodine Receptor at Serine 2808 Is Not Involved in Cardiac Dysfunction After Myocardial Infarction

Hongyu Zhang, Catherine Makarewich, Hajime Kubo, Wei Wang, Jason Duran, Ying Li, Remus Berretta, Walter J. Koch, Xiongwen Chen, Erhe Gao, Hector Valdivia and Steven R. Houser

Circ Res. published online February 2, 2012;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2012/02/01/CIRCRESAHA.111.255158

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2012/02/02/CIRCRESAHA.111.255158.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Detailed Methods

**Mice and Experimental Protocol**  WT and RyR-S2808A mice were used in these studies. The methods used to generate the RyR-S2808A mouse have been described previously. Experiments were performed based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the Temple University Committee on Animal Care. Myocardial infarction (MI) surgeries were carried out in mice at the age of 4 months. Mice were anesthetized with 2% isoflurane inhalation. A skin incision was made over the left thorax, and the pectoral muscles were retracted to expose the ribs. At the level of the fifth intercostal space, the heart was exposed and pumped out through an expanded space between ribs. A permanent knot or slipknot (for reperfusion purpose) was made around the left anterior descending coronary artery (LAD) 2–3 mm from its origin with a 6-0 silk suture. The heart was immediately placed back into the intrathoracic space after the knot was tied, followed by manual evacuation of pneumothoraces and closure of muscle and the skin suture by means of the previously placed purse-string suture. Sham-operated animals were subjected to the same surgical procedures except that the suture was passed under the LAD but was not tied.

**Determination of Left Ventricle Infarct Size After MI** After mouse was anesthetized; heart was quickly removed and frozen in a dish placed on dry ice for a few minutes. Then the heart was quickly sliced into eight 1.2-mm-thick sections perpendicular to the long axis of the heart. The sections were incubated in PBS containing 2% triphenyltetrazolium chloride (TTC; Sigma) at room temperature for 15 min and digitally photographed. TTC-negative staining area (infarcted myocardium) and total circumference of section were measured by NIH Image J software. Myocardial infarction size (expressed as percentage) was calculated using infarct length divided by total circumference of tissue sections.

**In-vivo Functional Analysis** (Echocardiography, ECHO) ECHO was performed with VisualSonics Velvo 770 machine which is specifically designed for mice and rats. Mice were anesthetized with 2% isoflurane initially and then 1% during the ECHO procedure. Hearts were viewed in the short-axis between the two papillary muscles and analyzed in M-mode. Parameters were to be measured offline (Velvo software) including end-diastolic diameter (EDD), end-systolic diameter (ESD), posterior wall thickness (PWT), and septal wall thickness (SWT) to determine cardiac morphological changes and ejection fraction (EF), heart rate and fractional shortening (FS).

**Histology** Animals were anesthetized by sodium pentobarbital (0.1ml/100g) and heparinized intravenously. Hearts were excised, trimmed off excess tissue, weighed, washed and then perfused with 10% buffered formalin. The fixed heart tissues were dehydrated, embedded in paraffin, sectioned at 5-μm thickness, and then perform Masson’s Trichrome staining, which was used to evaluate gross morphology, fiber integrity or fibrosis. Images were acquired using SPOTINSIGHT software (Diagnostic Instruments Ins.). 3 fields were captured from each tissue section per heart and blue area were automatically analyzed using Metamorph 6.1 software (Universal Image Corp.). Average blue area percentage was calculated across at least 3 independent hearts.

**Cellular Functional Analysis** Myocytes were isolated from sham and post-MI animals’ hearts at the end of 2-3 weeks to measure cellular fractional shortenting (FS), calcium
transients ([Ca^{2+}]_{i}) and L-type calcium current (I_{CaL}). All experiments were done a 35-37°C, in superfused myocyte chambers mounted on fluorescence equipped microscopes. All myocytes were characterized with the same series of experiments. The detailed methods are described as below.

**Myocyte Isolation**
Mice were anesthetized with sodium pentobarbital (0.1ml/100g). The heart was excised, weighed and cannulated on a constant-flow langendorff apparatus. The heart was digested by retrograde perfusion of normal tyrode solution containing 180 U/mL collagenase and (mM): CaCl2 0.02, glucose 10, HEPES 5, KCl 5.4, MgCl2 1.2, NaCl 150, sodium pyruvate 2, pH 7.4. When the tissue has softened, left ventricular tissue is gently minced, filtered, and equilibrated in Tyrodes solution with 0.2 mM CaCl2, and 1% bovine serum alumin (BSA) at room temperature. Routinely, our initial yield is >90% rod-shaped VMs, and >80% calcium-tolerant, rod-shaped VMS survive by the end of the isolation.

**I_{CaL} Measurement**
I_{CaL} was measured in a sodium-free and potassium-free solution. Isolated myocytes were placed in a chamber mounted on an inverted microscope (Nikon Diaphot) and perfused with 1mM calcium-containing Tyrode solution. Both the inflow solution and the chamber were water-heated to maintain the temperature at 36±1°C. A 4-5 MΩ pipette filled with a Cs+-containing solution [composition in mM: 130 Cs-aspartate, 10 N-methyl-Dglucamine (NMDG), 20 tetraethylammonium chloride, 10 HEPES, 2.5 Tris-ATP, 1 MgCl2, and 10 EGTA, pH 7.2] was used to obtain gigaseals. Once a gigaseal was formed, the patch was ruptured and the cell was dialyzed for 10 min. The extracellular bath was then changed to a 2mM calcium -containing Cs+ substitution bath solution (composition in mM: 2 4-aminopyridine, 2 CaCl2, 5.4 CsCl, 10 glucose, 5 HEPES, 1.2 MgCl2, and 150 NMDG, pH 7.4 with CsOH). Membrane voltage was controlled by an Axopatch 2A voltage-clamp amplifier and digitized by Digidata 1322 using pClamp8 software (Molecular Devices). Once the signal was converted to digital format, it was stored on a personal computer for off-line analysis with Clampfit 10 (Axon Instruments). The flow of the bathing solution was 2–3 ml/min.

**Fractional Shortening Magnitude, Ca^{2+} Transients and SR Load Measurements**
Fractional Shortening Magnitude and transients were measured simultaneously in myocytes incubated with Tyrodes solution containing 1mM Ca^{2+} at rates of 0.5 and 2 Hz stimulation with an edge detector. [Ca^{2+}]_{i} were measured in paced myocytes loaded with Fluo-4AM as described in previous studies. Once a steady state had been reached, 10 and 100nM Isoproterenol (Iso) was applied through the perfusion solution. Once a stable effect of Iso had been achieved, at least 20 continuous contractions and Ca^{2+} transients were recorded and averaged for analysis. Ca^{2+} transients were fit with a single-exponential decay function to determine the decay rate. To measure SR Ca^{2+} content, myocytes were paced at 0.5 Hz for 10 consecutive contractions, and 10 mM caffeine was then rapidly applied via a glass pipette close to the myocyte with a Pico spritzer. Since caffeine cannot be repetitively applied on the same cell, the SR Ca^{2+} content after 10nM Iso was measured by a caffeine spritz after stable effects of Iso on myocyte contraction and Ca^{2+} transients had been observed.

**In vivo hemodynamic measurements**
Mice were anesthetized with a 2% Isoflurane and the right common carotid artery was isolated and cannulated with 1.4 French micromanometer (Millar Instruments, Houston, TX). LV pressure, LV end-diastolic pressure (LVEDP) and heart rate (HR) were measured by this catheter advanced into
the LV cavity, and data was recorded and analyzed on a PowerLab System (AD Instruments Pty Ltd., Mountain View, CA). These parameters as well as maximal values of the instantaneous first derivative of LV pressure (+dP/dtmax, as a measure of cardiac contraction) and minimum values of the instantaneous first derivative of LV pressure (-dP/dtmin, as a measure of cardiac relaxation) were recorded at baseline and after administration of the β-adrenergic receptor (βAR) agonist, isoproterenol (Iso, 2ug/Kg and 2mg/Kg i.p. injection).

**Western Blotting**  Cytoplasmic and membrane protein were isolated from ventricular tissue using PBS lysis buffer containing: 0.5% Triton X-100, 5 mM EDTA (pH7.4), phosphatase inhibitors (10 mM NaF and 0.1 mM NaVO4), proteinase inhibitors (10 µg/ml aprotinin, 10 µg/ml leupeptin, 1mM phenylmethylsulfonyl fluoride, 5 µg/ml pepstatin A, 8 µg/ml calpain inhibitor I & II, and 200 µg/ml benzamidine). Cardiac actin was isolated from resulting pellet using PBS lysis buffer containing 2% sodium dodecyl sulfate, SDS (FisherBiotech), 1% IGEPAL CA-630 (Sigma), 0.5 % deoxycholate (Sigma), 5 mM EDTA (pH 7.4), and proteinase inhibitors. Protein abundance and phosphorylation levels in isolated protein were analyzed with Western blot analysis as described previously 8. Target antigens were probed with the following antibodies: phospholamban (PLB) (Upstate Biotechnology), RyR (Research Diagnostics), α-sarcomeric actin (Sigma), LTCC-α1C subunit (Chemicon), GAPDH (Serotec), PS2809-RyR, PS16-PLB, and PT17-PLB (Badrilla).

**Statistics**  Data are presented as mean±SEM. Between-group comparisons were performed by using the one way ANOVA or t-test. For all tests, statistical significance was set at P<0.05.
References


**Online Figure I** A. Representative images of cardiac tissue sections stained with TTC after 4 weeks MI. B. Representative images of fixed hearts and middle cross sections with infarct zone. C. Representative cardiac histology tissue sections with H&E staining after 4 weeks MI.
Online Figure II  Representative Western blots of Ca\(^{2+}\) regulatory proteins in heart tissues from WT and S2808A mice 4 weeks after sham or MI-operated procedure.
Online Figure III  

A. Voltage dependence of $I_{\text{Ca,L}}$ activation in sham WT (n=6) and S2808A myocytes (n=5) +/- Iso (1umol/L).  

B. Voltage dependence of $I_{\text{Ca,L}}$ activation in post-MI WT (n=6) and S2808A myocytes (n=5) +/- Iso (1umol/L).
Online Figure IV A-B. Representative traces of sham and post-MI myocyte contractions -/+ Iso (100nmol/L) at 0.5 Hz pacing rate from WT and S2808A hearts. C-D. Representative traces of sham and post-MI myocyte contractions -/+ Iso (100nmol/L) at 2 Hz pacing rate from WT and S2808A hearts.
Online Figure V  A-B. Representative traces of sham and post-MI myocyte Ca\textsuperscript{2+} transients +/- Iso (100nmol/L) at 0.5 Hz pacing rate from WT and S2808A hearts. C-D. Representative traces of sham and post-MI myocyte Ca\textsuperscript{2+} transients +/- Iso (100nmol/L) at 2 Hz pacing rate from WT and S2808A hearts.
Online Figure VI A. Cardiac function (dP/dT) with Isoproterenol treatment (2ug/Kg and 2mg/Kg i.p. injection) was determined by hemodynamics measurement using Miller catheterization. B. Peak dP/dT after Iso treatment was assessed in WT and S2808A mice.