Role of RyR2 Phosphorylation in Heart Failure and Arrhythmias

Protein Kinase A–Mediated Hyperphosphorylation of the Ryanodine Receptor at Serine 2808 Does Not Alter Cardiac Contractility or Cause Heart Failure and Arrhythmias

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Abstract: This Controversies in Research article discusses the hypothesis that protein kinase A (PKA)-mediated phosphorylation of the Ryanodine Receptor (RyR) at a single serine (RyRS2808) is essential for normal sympathetic regulation of cardiac myocyte contractility and is responsible for the disturbed Ca2+ regulation that underlies depressed contractility in heart failure. Studies supporting this hypothesis have associated hyperphosphorylation of RyRS2808 and heart failure progression in animals and humans and have shown that a phosphorylation defective RyR mutant mouse (RyRS2808A) does not respond normally to sympathetic agonists and does not exhibit heart failure symptoms after myocardial infarction. Studies to confirm and extend these ideas have failed to support the original data. Experiments from many different laboratories have convincingly shown that PKA-mediated RyRS2808 phosphorylation does not play any significant role in the normal sympathetic regulation of sarcoplasmic reticulum Ca2+ release or cardiac contractility. Hearts and myocytes from RyRS2808A mice have been shown to respond normally to sympathetic agonists, and to increase Ca2+ influx, Ca2+ transients, and Ca2+ efflux. Although the RyR is involved in heart failure–related Ca2+ disturbances, this results from Ca2+-calmodulin kinase II and reactive oxygen species–mediated regulation rather than by RyR2808 phosphorylation. Also, a new study has shown that RyRS2808A mice are not protected from myocardial infarction. Collectively, there is now a clear consensus in the published literature showing that dysregulated RyRs contribute to the altered Ca2+ regulatory phenotype of the failing heart, but PKA-mediated phosphorylation of RyRS2808 has little or no role in these alterations. (Circ Res. 2014;114:1320-1327.)

Key Words: heart failure ■ myocardial contraction ■ ryanodine receptor calcium release channel
Congestive heart failure (CHF) is a syndrome in which the heart is unable to pump an adequate amount of blood to properly support tissue metabolic needs during normal daily activities. Patients having this syndrome die prematurely, from either hemodynamic collapse secondary to poor cardiac pump function or from lethal arrhythmias. CHF is caused by a host of primary diseases including ischemic heart disease, hypertension, and genetic abnormalities. The failing heart is usually dilated and pumping against excessive systemic pressures (hypertension). Both conditions require the diseased heart to generate a greater than normal systolic wall stress. Therefore, myocytes within the failing heart must generate greater than normal forces to eject a normal or even a reduced cardiac output. Persistently high levels of neuroendocrine (primarily sympathetic) signaling to the failing myocytes cause them to generate this high wall stress. These regulatory systems are recruited in normal individuals on an intermittent basis to increase cardiac contractility in times of need, such as during exercise. In CHF, these same systems are required to maintain basal function, and this reduces contractility reserve. Enhanced sympathetic activity is a central feature of CHF, and patients with CHF are dependent on this hyperadrenergic state to maintain blood pressure and cardiac output. Myocytes in the failing heart structurally (hypertrophy) and functionally remodel in response to their excessive work demands. There are significant alterations in systolic and diastolic Ca2+, and adrenergic regulation of contractile Ca2+ is deranged. Interestingly, while failing myocytes are forced to develop greater than normal force in vivo, when they are isolated from failing systems, they have smaller, prolonged contractions and smaller and lower force in vitro. Failure of the failing heart is largely responsible for the normal effects of sympathetic agonists on cardiac contractility in times of need, such as during exercise. In CHF, these same systems are required to maintain basal function, and this reduces contractility reserve. Enhance sympathetic activity is a central feature of CHF, and patients with CHF are dependent on this hyperadrenergic state to maintain blood pressure and cardiac output.

Myocytes in the failing heart structurally (hypertrophy) and functionally remodel in response to their excessive work demands. There are significant alterations in systolic and diastolic Ca2+, and adrenergic regulation of contractile Ca2+ is deranged. Interestingly, while failing myocytes are forced to develop greater than normal force in vivo, when they are isolated from failing hearts and compared with normal myocytes under identical conditions they have smaller, prolonged contractions and smaller and more slowly decaying Ca2+ transients. Changes in the abundance and phosphorylation state of molecules that regulate contractile Ca2+, including L-type Ca2+ channels (LTCCs), the sarco/ endoplasmonic reticulum ATPase (SERCA), the SR regulatory protein phospholamban (PLN), the Ca2+ release channel (ryanodine receptor, RyR), and the Na/Ca exchanger (NCX), underlie the changes in contractile Ca2+ seen in human heart failure. Restoring normal myocyte Ca2+ regulation is one possible strategy for improving cardiac function in heart failure. In this Controversies article, I discuss the evidence for the idea that PKA-mediated phosphorylation of a single amino acid (Serine2808) on RyR (RyRS2808) is largely responsible for sympathetic regulation of myocyte contractility and for deranged Ca2+ regulation in the failing heart.

Standard of care CHF therapies provide some clinical benefit to patients with CHF, but there is still a tremendous need to develop novel therapies to improve the pump function of the failing heart without inducing lethal arrhythmias. Obviously a strategy that makes stronger myocytes would produce a stronger heart with better pump function. However, the development of CHF inotropic therapeutics has been challenging, and clinical trials using novel inotropic therapies have largely failed. It is important to again point out that the myocytes within the failing heart are already developing higher than normal force (against the pathologically increased wall stress), so inotropic therapies designed to increase myocyte contractility even further might induce the excess levels of [Ca2+] that cause cell death and arrhythmias, possibly making things worse rather than better. The important point here is that there is a need to identify and test novel targets for heart failure therapeutics.

In 2000, the Marks laboratory identified a potential target for inotropic therapy in heart failure. This group published a series of exciting studies suggesting that excess (hyper) activation of sympathetic signaling cascades caused PKA-mediated hyperphosphorylation of RyR at a single amino acid (serine 2808). The results presented in these studies strongly support the hypothesis that hyperphosphorylation of RyRS2808 is largely responsible for the contractility defects of the failing myocyte. Results from these studies also provided compelling data showing that preventing PKA-mediated RyRS2808 hyperphosphorylation, while leaving all other PKA and Ca2+-calmodulin kinase (CaMKII) targets unmodified, significantly improves cardiac function after myocardial infarction and prevents heart failure in a genetically modified mouse. These were exciting and provocative findings.

The molecular mechanism by which RyRS2808 phosphorylation was thought to produce these effects was carefully documented in these studies. It was shown that PKA-mediated phosphorylation of RyRS2808 increases the Ca2+ binding affinity to RyR to enhance RyR opening probability. This effect was produced by displacement of a stabilizing protein, FK-binding protein 12.6 (FKBP12.6), from RyR. Because 4 RyR monomers oligomerize to form the Ca2+ release channel in the SR, there are 4 of these PKA sites per functional molecule. The idea proposed was that phosphorylation of 1 or 2 of these sites is responsible for PKA-mediated increases in SR Ca2+ release (normal contractility regulation by the sympathetic nervous system), and phosphorylation of 3 or 4 sites destabilized the molecule and caused what has since been termed SR Ca2+ leak. When S2808 was replaced with a nonphosphorylatable amino acid (alanine), RyRS2808A mice did not respond normally to adrenergic agonists, and they were resistant to pathological stressors that cause CHF. These data strongly support the idea that RyR phosphorylation at a single amino acid is largely responsible for the normal effects of sympathetic agonists on cardiac contractility and for contractility defects in heart failure. The RyRS2808A post-myocardial infarction (MI) data suggested that preventing RyRS2808 hyperphosphorylation is a viable approach to treat patients with heart failure.
the normal and diseased heart, and can competent investigators in the field validate the results? In my opinion the answers to these questions are no and no.

I have been actively studying fundamental aspects of cardiac contractility in health and disease for 30+ years, and studies performed in the my laboratory have helped define the cellular and molecular bases of normal cardiac excitation–contraction (EC) coupling and contractility regulation and have helped to define those changes in myocyte Ca2+ regulation in myocytes from failing human hearts that reduce contractility reserve.

In this Controversies in Research article I try to explain why, in my view, alterations in the properties of RyR via PKA-mediated phosphorylation are unlikely to play any important role in the regulation of normal myocyte contractility and review the substantial evidence against any significant role for PKA-mediated phosphorylation of RyRS2808 in heart disease.

EC coupling is now well understood in the heart, and the specific roles of critical molecular participants are clearly defined. Based on our understanding of normal EC coupling, the RyR phosphorylation hypothesis makes little or no sense to me. Cardiac myocyte RyRs are essential for normal EC coupling, and their role is to provide a pathway for Ca2+ to move from the SR (where it is stored) into the bulk cytoplasm to activate the contractile proteins. RyRs are primarily localized to the SR membranes that are closely associated with LTCCs within the transverse (T) tubules, invaginations of the surface membrane. The close proximity of LTCCs in the plasma membrane and RyRs in the junctional SR is essential for the high fidelity of the Ca2+-induced SR Ca2+ release that results in the contractile Ca2+ transient. The gating (opening) of RyRs in the heart is controlled by the [Ca2+]i within the junctional cleft between the T-Tubule and associated SR membranes and by the [Ca2+]i within the SR lumen. In resting myocytes (diastole in the normal heart), Ca2+ is sequestered into the SR lumen (via the SR Ca2+ATPase, SERCa), and the RyR is closed because the [Ca2+]i in the junctional cleft is below the threshold for the Ca2+-mediated RyR opening during normal EC coupling, and [Ca2+]i within the SR lumen is below the high level needed to induce spontaneous opening of RyRs. With each cardiac action potential, a fraction of the voltage-dependent LTCCs open, and Ca2+ enters the diffusion limiting junctional cleft. The elevation in cleft [Ca2+] promotes Ca2+ binding to RyRs and causes RyR opening. The opening of a single RyR channel, and the resultant Ca2+ flux, provides a sufficient rise in cleft [Ca2+] to cause the opening of other RyRs within the junctional complex (couplon). The result is regenerative, local Ca2+ release in an individual couplon. Importantly, it is established that there is sufficient LTCC Ca2+ entry during every heart beat to cause the regenerative release of SR Ca2+ from all couplons in a normal myocyte. The established literature shows that nature has designed regenerative SR Ca2+ release within each couplon to have a large safety factor (more than enough Ca2+ influx to induce the locally regenerative release process) to ensure that EC coupling does not fail. It seems to me that nature has designed a system that ensures that a process as important as EC coupling in the normal heart is not poised at the brink of failure. The point here is that RyRs are involved in a regenerative rather than in a graded process.

In normal individuals, cardiac contractility (the force of cardiac contraction) must be varied over a wide range, to produce cardiac outputs that are proportional to the ever-changing metabolic demands of the body. A control system that allows for an analog form of contractility regulation is essential for normal heart function. PKA-mediated RyRS2808 phosphorylation is unlikely to be involved in this type of continuously graded regulation of contractility because RyRs are digital molecules (they are either opened or they are closed) and they are involved in a locally regenerative (digital) process. Stated a bit differently, RyR-dependent SR Ca2+ release either happens or it does not happen, and because all (or almost all) couplons are recruited with each normal heart beat, regulating this process will not be an effective mechanism to vary the contractility of the heart. Stated still a third way, contractility is NOT varied by regulating the number of couplons that release their stored Ca2+. Varying the amount of Ca2+ released at each couplon regulates contractility.

One of my major problems with the RyR phosphorylation hypothesis is that it fails to provide an explanation for how sympathetic mediated phosphorylation of the RyRs involved in locally regenerative SR Ca2+ release can produce graded changes in myocyte contractility. Therefore, even if phosphorylation of RyRS2808 increases Ca2+-dependent RyR opening (a debatable topic on its own right), there would be no alterations in the number of couplons involved in release. Modeling and direct experimental evidence do not support the idea that changing RyR Ca2+-dependent opening has a significant effect on SR Ca2+ release and the amplitude of the systolic Ca2+ transient.

Importantly, the molecular mechanisms that produce sympathetic regulation of cardiac contractility have been thoroughly studied and can be fully explained by the well-known and universally observed effects of β-adrenergic agonists on LTCCs and the SERCa regulatory protein PLN. PKA-mediated phosphorylation of the LTCC complex increases channel opening probability, and the net result is an increase in Ca2+ entry that is graded in proportion to the sympathetic response. These changes have no significant effect on the number of couplons that release their stored [Ca2+] at because there is already sufficient LTCC-mediated Ca2+ entry under basal conditions to fully activate EC coupling. However, the releasable SR Ca2+ stores are increased by sympathetic activity via the additional LTCC Ca2+ influx, to produce graded increases in the SR Ca2+ load. The SR Ca2+ release and SR Ca2+ loading aspects of Ca2+ entry through the LTCC have been well known for decades.

PLN is an inhibitor of SERCa and, in the absence of sympathetic agonists, it restrains SERCa Ca2+ transport. PKA-mediated PLN phosphorylation at serine 16 removes the inhibitory effect of PLN on SERCa, resulting in enhanced SR Ca2+ uptake. β-adrenergic agonists therefore increase Ca2+ entry and stimulate Ca2+ uptake by the SR to grade the amount of releasable Ca2+ stored in the SR. The well-established mechanism for grading cardiac contractility is by sympathetic stimulation of Ca2+ influx and SERCa to grade the releasable Ca2+ stores in the SR. No role for regulation of RyR open probability is needed to explain the normal regulation of cardiac contractility, and, as explained above, this idea conflicts with the evidence collected by a host of laboratories during many decades.
Our group participated directly in the studies that show that PKA-mediated phosphorylation of RyRS2808 has no effect on cardiac contractility. To explore this topic, we used an RyRS2808A (with no PKA-mediated RyRS2808 phosphorylation) knockin mouse kindly provided by the Valdivia laboratory.50 Our experiments clearly showed that the RyRS2808A heart (in vivo and in vitro) and isolated RyRS2808A myocytes respond normally to β-adrenergic agonists.32 Our results showed that the lack of catecholamine induced RyRS2808 phosphorylation had no effect on EC coupling or the regulation of cardiac contractility, in line with the findings of the Bers laboratory.49 The Marks laboratory recently re-explored this issue and published different results, in favor of a role for RyRS2808 phosphorylation in the regulation of normal myocyte contractility.32 Their study showed that catecholamines failed to induce normal increases in heart rate, in vivo contractility or in vitro increases in [Ca2+] transients and contraction in RyRS2808A myocytes. Their conclusion was that eliminating the effect of RyRS2808 phosphorylation on Ca2+-dependent opening of Ry abolishes β-adrenergic-mediated increases in contractility. Given my discussion of basic EC coupling above, it is unclear how these effects could actually come about. In my view, the differences between the results of the 2 groups are not easily explained.

One of the issues that was inadequately explored in the study from the Marks laboratory is that β-adrenergic effects on the LTCC and PLN should have been intact in the RyRS2808A mouse. Therefore, Ca2+ influx and SR Ca2+ loading should have increased with catecholamine stimulation. Unfortunately, these critical parameters were never measured.32 Where did this extra Ca2+ influx go? It is well established that when Ca2+ influx increases in cardiac myocytes there must be an increase in the counterbalancing Ca2+ efflux, to maintain the new steady state.55 This is established, fundamental, normal physiology. If increased Ca2+ influx is not met with the required increase in Ca2+ efflux, myocytes will Ca2+ overload and die. Our results clearly document that catecholamines increase LTCC-mediated Ca2+ entry and increase SR Ca2+ load to increase SR Ca2+ release in both normal and RyRS2808A myocytes. The resultant increase in the systolic Ca2+ transient increases Ca2+ efflux through the NCX and brings about flux balance in the new steady state.55 How catecholamines could fail to cause those increases in the systolic [Ca2+] transient required to produce Ca2+ flux balance in the RyRS2808A myocytes was not explained in the studies from the Marks laboratory.32 There is no reason to think that catecholamines failed to increase Ca2+ influx through the LTCC and Ca2+ uptake by the SR. It is unclear how a new flux balance was achieved? Because these critical issues were not adequately examined, I assume that these RyRS2808A mice either do not have catecholamine-dependent regulation of the LTCC or Ca2+ was transported out of myocytes via a novel mechanism that does not require an increase in the systolic Ca2+ transient.

There is one area of agreement in all studies with RyRS2808A mice. This mouse has no basal phenotype.30,32,33,48,50,51 The RyRS2808A mouse has normal basal contractility both in vivo and in vitro. This is an unexpected finding if you think that this single amino acid is essential for the moment-to-moment regulation of myocyte contractility.32 So how does an RyRS2808A mouse, which requires phosphorylation at RyRS2808 for the regulation of cardiac contractility function normally? The answer must be adaptation of other related processes, which the mouse is really good at. Therefore, we examined the idea that the absence of a basal phenotype was the result of adaptive changes in other Ca2+ regulatory proteins.51 However, we found no changes in the abundance or phosphorylation state of any relevant Ca2+ regulatory protein in the RyR S2808A mouse.31 In my opinion, the loss of an essential Ca2+ regulatory mechanism should have induced adaptive changes in other related Ca2+ regulatory processes. I form this opinion based on results of others who have shown that when a critical protein involved in myocyte Ca2+ regulation is eliminated or disrupted, there are major adaptive changes in other components of Ca2+ regulation.56,57 An example is when the major Ca2+ efflux mechanism, the NCX, is conditionally deleted (in most myocytes). This conditional NCX-deficient mouse adapts by significantly reducing the expression of the major Ca2+ influx pathway, through the LTCC.55 The mouse with forced reduction in Ca2+ efflux capacity immediately adapts by reducing Ca2+ influx. Basal state function is fairly well preserved but, with consequences, the animal is stress intolerant.57 Our studies30,51 failed to show any alterations in the adrenergic regulation of Ca2+ current, EC coupling, myocyte [Ca2+] transients and contractions, and in vivo or in vitro heart function in the RyRS2808A mouse. Our interpretation of these findings is that there were no adaptations because there is no role for RyRS2808 in the regulation of cardiac contractility. The Marks group has tried to explain away our findings by suggesting that we used excessively high catecholamine concentrations that somehow masked differences between WT and RyRS2808A myocytes. Unfortunately, the Marks group32 misquoted aspects of our published work.51 The facts are that we used a 10-fold lower (10 nmol/L) isoproterenol concentration than that used in their study32 of isolated RyRS2808A hearts (100 nmol/L). We found no differences in WT and RyRS2808A mice. Interested readers should compare the raw and average data reported in the isolated heart experiments published in these 2 studies32,51 and form your own opinion. Just to double check, we also re-explored the idea that defective catecholamine effects on isolated myocyte contractility were only observed at high concentrations, and we found absolutely identical catecholamine responsiveness in normal and RyRS2808A myocytes, over a broad range of isoproterenol (ISO) concentrations.50 Our results support the idea that PKA-mediated RyR regulation has no significant influence on sympathetic mediated regulation of cardiac function in the normal heart. As we have stated previously,50 there is the unlikely possibility that the 2 RyRS2808A mice have fundamentally different properties. Obviously this could be tested if both mouse lines were freely shared.

This brings us to a discussion of the hypothesis that PKA-mediated hyperphosphorylation of RyRS2808 is critical to contractile defects, heart failure progression, and arrhythmias in human disease.54 Based on the discussion above, readers will not be surprising that I think this is unlikely to be an important mechanism of heart failure induction or progression. However, I should also state that there is overwhelming evidence that SR function is disrupted in heart failure and that alterations in the behavior of the RyR are involved in this disruption. The issue
being specifically discussed here, however, is whether PKA-mediated hyperphosphorylation of RyRS2808 is singularly responsible for altered RyR function in the failing heart. Although I think there is substantial evidence that RyRS2808 phosphorylation is not involved in CHF contractility defects (see below), I am in agreement with those who have shown alterations in RyR function produced by CaMKII-mediated RyRS2814 phosphorylation\(^8\) by oxidative stress pathways\(^{40}\) or by mutations that are known to induce lethal ventricular arrhythmias.\(^{61}\) These studies clearly show that disease- or mutation-specific alterations in RyR function are involved in SR Ca\(^{2+}\) leak\(^2\) and catecholaminergic polymorphic ventricular tachycardia.\(^{63}\)

The data published in support of the PKA-mediated RyRS2808 hypothesis\(^{16,18-20,22,33,64}\) show that (1) PKA-mediated RyRS2808 is hyperphosphorylated in heart failure; (2) RyRS2808 hyperphosphorylation causes FKBP12.6 to dissociate from RyR, and this destabilizes RyR and reduces the \([\text{Ca}^{2+}]\) needed to induce RyR opening; (3) hyperphosphorylation of RyR at other PKA sites or by CaMKII is not present in CHF; and (4) eliminating hyperphosphorylation at RyRS2808 (RyRS2808A) improves cardiac function after myocardial infarction. Given the strength of the data in these publications, it would seem that PKA-mediated hyperphosphorylation of RyRS2808 is a critical abnormality in heart failure, and that correction/prevention of this single phosphorylation event will significantly improve cardiac function with disease stress. I will again apply simple standards to the evaluation of these findings. Have others been able to confirm these results, and do the results make any sense with respect to what we know about defects in Ca\(^{2+}\) regulation in heart failure? In my view, the vast majority of others who have explored these issues have not been able to confirm the results from the Marks group (see below).

**Is RyRS2808 Hyperphosphorylated in Human Heart Failure?**

There is no doubt that persistent sympathetic nervous system activity is required to maintain the pump function of the failing heart. Even with the known downregulation of adrenergic signaling cascades, there is still persistent activation of \(\beta\)-adrenergic signaling cascades, and this should promote RyRS2808 phosphorylation. The original hyperphosphorylation report clearly documents altered RyRS2808 phosphorylation.\(^{16}\) However, this has not been a universal finding.\(^{59,65}\) In fact, a recent report from the Wehrens laboratory\(^{66}\) did not find any RyRS2808 hyperphosphorylation in any form of human heart failure. Interestingly, the Marks group has shown that when heart failure animals are treated with \(\beta\)-blockers, RyRS2808 phosphorylation returns to normal, although the heart failure state was still present (tachypacing model of CHF).\(^{20}\) Because \(\beta\)-blockers are standard of care for patients with heart failure, and these patients still have heart failure while they are treated with these drugs, it could be that the patients in the Wehrens study were being treated with \(\beta\)-blockers and this normalized their RyRS2808 phosphorylation state. My personal view is that every PKA (and CaMKII) phosphorylation site is altered in human heart failure, regardless of standard of care therapy, because adrenergic and Ca\(^{2+}\) stress is persistent in this syndrome. In addition, I always interpret studies with explanted failing human tissues with great caution because major changes in protein post-translational modification are present when tissue is not protected from ischemic injury during surgical explantation of failing hearts, using techniques pioneered by Ken Margulies.\(^7\) I cannot find a systematic study of S2808 and S2814 phosphorylation states in various forms of human heart failure where the \(n\) is sufficient to make a meaningful statement about this issue. However, given what we know about human heart failure, I think there is every reason to consider that RyRs in failing myocytes have significant post-translational modifications including phosphorylation changes at S2808.

**Does RyRS2808 Hyperphosphorylation Cause FKBP12.6 to Dissociate From RyR and Reduce the \([\text{Ca}^{2+}]\) Needed to Induce RyR Opening?**

The Bers laboratory has thoroughly explored this hypothesis and has presented compelling data showing that FKBP12.6 is strongly bound to the RyR and is not displaced by PKA phosphorylation.\(^{66}\) The Bers laboratory\(^{66,67}\) and others\(^{68-70}\) have also published results showing that CaMKII or \(\gamma\)-nitrosylation rather than PKA-mediated phosphorylation of RyR causes changes in RyR function that enhances Ca\(^{2+}\) sparks (local Ca\(^{2+}\) release induced by local SR Ca\(^{2+}\) overload) and SR Ca\(^{2+}\) leak. I think that the weight of existing evidence suggests that PKA-mediated phosphorylation of RyRS2808 has no significant effect on RyR function. The original data supporting this hypothesis have not been adequately confirmed.

The original reports supporting the RyRS2808 hypothesis provided strong experimental data showing that hyperphosphorylation of RyR at other PKA sites or at CaMKII sites (S2814) does not occur in CHF.\(^{30}\) These data have not been confirmed by others, and there is now overwhelming data showing that CaMKII\(^{58,59,66,67,69,70}\) and oxidative stress\(^6\) are major regulators of RyR function in disease. There are many published studies clearly documenting the benefits of CaMKII inhibition in a wide range of heart failure models (MI, calcium neuron overexpression, isoproterenol, chronic and severe thoracic aortic constriction) and preheart failure models (acute or mild thoracic aortic constriction, angiotensin II, aldosterone, hypertrophy, ischemia reperfusion injury; reviewed by Luo et al\(^{36}\)). I apologize to the many investigators I have not been able to quote who have dispelled the idea that PKA is involved in disease-related alterations in RyR function. These studies have almost universally shown that activation of CaMKII is the critical factor that culminates in what has been termed SR Ca\(^{2+}\) leak in disease. In this regard, the Wehrens laboratory has published reports\(^{27}\) dispelling portions of idea that CaMKII was not involved in RyR dysfunction in CHF.\(^{30}\) Curiously, his most recent data suggest that CaMKII phosphorylation of RyRS2814 is only involved in SR dysfunction in pressure overload–induced hypertrophic cardiomyopathy,\(^65\) and does not contribute to ischemic cardiomyopathy, consistent with his earlier work in the mouse.\(^{35}\) The idea that CaMKII is not activated in CHF induced by ischemic heart disease seems highly unlikely.\(^54\) It is abundantly clear that myocytes from patients with ischemic heart failure have significant Ca\(^{2+}\) stress, as in all other forms of CHF. How RyRs escape Ca\(^{2+}\) and ROS-mediated activation\(^{68-70}\) in ischemic heart failure has not been adequately explained and is inconsistent with
published data.\textsuperscript{58} The notion that RyRS2808 phosphorylation is involved in RyR modifications in ischemic but not hypertrophic heart disease also does not fit with a host of previous studies.\textsuperscript{58,65} The abundance of existing data strongly supports the idea that CaMKII is pathologically activated (autophosphorylated and oxidized) in all forms of human heart failure and that CaMKII-mediated phosphorylation of RyRS2814 should be present in ischemic cardiomyopathy.\textsuperscript{58}

The most exciting aspect of the original RyR hypothesis was that preventing PKA-mediated phosphorylation of RyRS2808 prevented critical aspects of cardiac dysfunction after myocardial infarction.\textsuperscript{30,31} As I have stated above, the idea that a single PKA phosphorylation site is responsible for the complex contractility defects in CHF does not seem logical, especially given the evidence that this molecule is not involved in the regulation of normal cardiac contractility. However, this idea is still being promoted.\textsuperscript{44} Given my laboratories long interest in the bases of contractility defects in human heart failure, I felt compelled to see whether my group could confirm the findings that RyRS2808A knockin mice are protected from cardiac dysfunction after MI. Dr Hector Valdivia again was kind enough to share his mice\textsuperscript{46} with us. He had already shown that RyRS2808A mice were not protected from pressure overload--induced alterations in cardiac structure and function.\textsuperscript{48} We then performed a complementary study in which RyRS2808A mice were subjected to MI,\textsuperscript{50} so that both pressure overload and ischemic stress were both studied. Consistent with the Valdivia results, we found the RyRS2808A failed to protect the heart from MI-induced structural and functional remodeling. In addition, we showed that the effects of isoproterenol (at low and high concentrations) had identical effects on myocyte function in wild-type and RyRS2808A mice/myocytes before and after MI.\textsuperscript{50}

The Wehrens laboratory has recently found no change in RyRS2808 phosphorylation in the failing human heart (any pathogenesis) and has shown that RyRS2814A fails to protect mouse hearts after MI.\textsuperscript{65} As discussed above, our study shows that RyRS2808A also fails to protect the heart after MI.\textsuperscript{50} Therefore, the published data with phosphorylation deficient RyR mice would suggest that RyR phosphorylation at either S2808 or S2814 has little to do with contractile deficits in the heart after MI, at least in the mouse.

In summary, the original reports that PKA-mediated RyRS2808 phosphorylation is responsible for normal adrenergic regulation of cardiac contractility and for contractility defects in disease has sparked significant research in the field. Many have tested this hypothesis and, collectively, these studies (from many independent laboratories) have been unable to find any important role for RyRS2808 phosphorylation in the regulation of cardiac contractility in health or disease. However, in the process of dispelling the PKA-mediated RyRS2808 phosphorylation hypothesis, the field has carefully explored RyR function in health and disease. These studies clearly show that RyR function is altered in disease and that this RyR dysfunction is linked to disturbed SR Ca\textsuperscript{2+} regulation, altered myocyte contractility reserve, and arrhythmias. A vast literature has evolved showing that CaMKII-mediated RyR phosphorylation and aberrant RyR s-nitrosylation are validated contributors to abnormal RyR behavior in disease.\textsuperscript{58}

The next step will be to determine whether pharmacological agents that eliminate CHF-induced RyR functional alterations make things better or worse. A parting thought is that maybe SR Ca\textsuperscript{2+} leak is a good thing in the Ca\textsuperscript{2+} stressed, failing heart? Maybe leak reduces the SR Ca\textsuperscript{2+} overload that promotes myocyte death signaling or enhances arrhythmias? Time and unbiased, high quality science will tell.

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### Disclosures

None.

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We appreciate this counterpoint article written by Dr Houser, who has made important contributions to the fields of excitation–contraction coupling and calcium signaling in healthy and failing hearts. We think that together our 2 counterpoint review articles provide a comprehensive overview of the present literature on ryanodine receptor type-2 (RyR2) phosphorylation. In our opinion, phosphorylation represents one of the major post-translational mechanisms through which RyR2-mediated sarcoplasmic reticulum (SR) Ca$^{2+}$ release is fine-tuned in the heart. Also, we agree that prominent research groups using seemingly similar models, experimental approaches, and reagents arrived at different conclusions regarding the potential role of RyR2-S2808 phosphorylation in both healthy and failing hearts, which is currently not fully explained.

In our companion article, we highlighted potential reasons for discrepant findings, which is not unusual in science. One critical issue seems to be that principal investigators of opposing articles employed different null hypotheses, which might have led to differential interpretation of the findings. For example, Benkusky et al. reported a significantly higher fractional shortening, which is a complex read-out signal, in RyR2-S2808A mice at 11 weeks after transverse aortic constriction, but the authors concluded that "Ablation of this site offers little protection during chronic stress, implying a limited role for RyR2-S2808 phosphorylation in the pathogenesis of HF."

Whereas we agree with Dr Houser that there remains controversy regarding the role of RyR2-S2808 phosphorylation in heart failure development, we caution against completely dismissing the idea that S2808 phosphorylation is possibly involved. For example, there is still insufficient detailed information about RyR2-S2808 phosphorylation–mediated regulation of Ca$^{2+}$-handling in humans.
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