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# Controversies in Cardiovascular Research:

## Role of Protein Kinase A Mediated Hyperphosphorylation of the Ryanodine Receptor at Serine 2808 in Heart Failure and Arrhythmias

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## Abstract

This “Controversies in Research” article discusses the hypothesis that protein kinase A 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 $Ca^{2+}$ regulation that underlies depressed contractility in heart failure. Studies supporting this hypothesis have associated “hyper” phosphorylation 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 (MI). 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 (SR) $Ca^{2+}$ release or cardiac contractility. Hearts and myocytes from RyRS2808A mice have been shown to respond normally to sympathetic agonists, and to increase $Ca^{2+}$ influx, $Ca^{2+}$ transients, and $Ca^{2+}$ efflux. While the RyR is involved in heart failure related $Ca^{2+}$ disturbances, this results from CaMKII and reactive oxygen species mediated regulation rather than by RyR2808 phosphorylation. Also, a new study has shown that RyRS2808A mice are not protected from MI. Collectively, there is now a clear consensus in the published literature showing that dysregulated RyRs contribute to the altered $Ca^{2+}$ regulatory phenotype of the failing heart, but PKA-mediated phosphorylation of RyRS2808 has little or no role in these alterations.

### Keywords

Ryanodine Receptor; $Ca^{2+}$ regulation; contractility; heart failure

The purpose of this “Controversies in Research” article is to discuss the evidence that dispels the hypothesis that protein kinase A (PKA) mediated phosphorylation of the Ryanodine Receptor (RyR; $Ca^{2+}$ release channel in the sarcoplasmic reticulum (SR)) at a single amino acid (Serine at position 2808; RyRS2808) is essential for normal sympathetic

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regulation of cardiac contractility and “hyper” phosphorylation of this same site causes depressed contractility reserve in heart failure.

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 suffering from this syndrome die prematurely, from either hemodynamic collapse secondary to poor cardiac pump function or from lethal arrhythmias[1]. CHF is caused by a host of primary diseases including ischemic heart disease, hypertension and genetic abnormalities. The failing heart is usually dilated and/or pumping against excessive systemic pressures (hypertension).Both conditions require the diseased heart to generatea greater than normal systolic wall stress. Therefore, myocytes within the failing heart must generategreater than normal forces to eject a normal or even a reducedcardiac output. Persistently high levels of neuroendocrine (primarily sympathetic) signaling to the failing myocytes causes 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”[2]. Enhanced sympathetic activity is a central feature of CHF, and CHF patients are dependent upon 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 $Ca^{2+}$ and adrenergic regulation of contractile $Ca^{2+}$ 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 to normal myocytes under identical conditions they have smaller, prolonged contractions and smaller and more slowly decaying$Ca^{2+}$ transients[3]. Changes in the abundance and phosphorylation state of molecules that regulate contractile $Ca^{2+}$, including L-type $Ca^{2+}$ channels (LTCCs), the sarcoplasmic reticulum ATPase (SERCa), the SR regulatory protein phospholamban (PLN), the $Ca^{2+}$ release channel (ryanodine receptor, RyR), and the Na/Ca exchanger, underlie the changes in contractile $Ca^{2+}$ seen in human heart failure[2–11]. Restoring normal myocyte $Ca^{2+}$ regulation is one possible strategy for improving cardiac function in heart failure. In this “Controversies”article I discuss the evidence for the idea that protein kinase A (PKA) mediated phosphorylation of a single amino acid (Serine2808) on RyR (RyRS2808) is largely responsible for sympathetic regulation of myocyte contractility and for deranged $Ca^{2+}$ regulation in the failing heart.

Standard of care CHF therapies provide some clinical benefit to CHF patients, but there is still a tremendous need to develop novel therapies to improve the pump function of the failing heart without inducing lethal arrhythmias[12]. 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[13, 14]. 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 toincrease myocyte contractility even further might induce the excess levels of [$Ca^{2+}$] that cause cell death[15] 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)[16–31]. The results presented in these studies strongly support the hypothesis that hyperphosphorylation of RyRS2808is 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 $Ca^{2+}$-Calmodulin Kinase (CaMKII) targets unmodified, significantly improves cardiac function after myocardial infarction and prevents heart failure in a genetically modified mouse[30]. These were exciting and provocative findings.

The molecular mechanism by which RYR-S2808 phosphorylation was thought to produce these effects was carefully documented in these studies[16, 18–33]. It was shown that PKA mediated phosphorylation of RyRS2808 increases the $Ca^{2+}$ binding affinity to RyR to enhance RyR opening probability. This effect was produced by displacement of a stabilizing protein, FKBP12.6 from RyR. Since 4 RyR monomers oligomerize to form the $Ca^{2+}$ release channel in the sarcoplasmic reticulum (SR) there are 4 of these PKA sites per functional molecule. The idea proposed was that phosphorylation of one or two of these sites is responsible for PKA mediated increases in SR $Ca^{2+}$ 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 $Ca^{2+}$ “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 induce 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[32] and for contractility defects in heart failure[33].The RyRS2808Apost MI data suggested that preventing RyRS2808 hyperphosphorylation is a viable approach to treat patients with heart failure!

As an active researcher in this field for many decades I was immediately interested in these results. I read the reports and asked my standard questions about the findings; do the results make sense with respect to what we know about the biology of 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[34–37] and have helped to define thosechanges in myocyte $Ca^{2+}$ regulation in myocytes from failing human hearts that reduce contractility reserve[4–8, 38–42].

In this *Controversiesin 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 very well understood in the heart and the specific roles of critical molecular participants are clearly defined[43, 44]. 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 $Ca^{2+}$ 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 L-type $Ca^{2+}$ channels (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 $Ca^{2+}$-induced SR $Ca^{2+}$ release that results in the contractile $[Ca^{2+}]$ transient. The gating (opening) of RyRs in the heart is controlled by the $[Ca^{2+}]$ within the junctional cleft between the T-Tubule and associated SR membranes and by the $[Ca^{2+}]$ within the SR lumen[43]. In resting myocytes (diastole in the normal heart) $Ca^{2+}$ is sequestered into the SR lumen (via the SR CaATPase, SERCa) and the RyR is closed because the $[Ca^{2+}]$ in the junctional cleft is below the threshold for the $Ca^{2+}$-mediated RyR opening during normal EC coupling, and $[Ca^{2+}]$ within the SR lumen is below the high level needed to induce spontaneous opening of RyRs[45]. With each cardiac action potential a fraction of the voltage dependent LTCCs open and $Ca^{2+}$ enters the diffusion limiting junctional cleft. The elevation in cleft $[Ca^{2+}]$ promotes $Ca^{2+}$ binding to RyRs and causes RyR opening. The opening of a single RyR channel, and the resultant $Ca^{2+}$ flux, provides a sufficient rise in cleft $[Ca^{2+}]$ to cause the opening of other RyRs within the junctional complex (couplon). The result is regenerative, local $Ca^{2+}$ release in an individual“couplon”. Importantly, it is established that there is sufficient LTCC $Ca^{2+}$ entry during every heart beat to cause the regenerative release of SR $Ca^{2+}$ from all couplons in a normal myocyte[46]. The established literature shows that nature has designed regenerative SR $Ca^{2+}$ release within each couplon to have a large safety factor (more than enough $Ca^{2+}$ influx to induce the locally regenerative release process) to ensure that EC coupling does not fail. It appears to me that nature has designed a system 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 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 $Ca^{2+}$ release either happens or it doesn’t happen, and since 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 $Ca^{2+}$. Varying the amount of $Ca^{2+}$ 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 $Ca^{2+}$ release can produce graded changes in myocyte contractility. Therefore, even if phosphorylation of RyRS2808 increases $Ca^{2+}$ dependent RyR opening (a debatable topic on its own right)[47, 48] there would be no alterations in the number of couplons involved in release. Modeling[44]and direct experimental evidence[49]does not support the idea that changing RyR $Ca^{2+}$ dependent opening has a significant effect on SR $Ca^{2+}$ release and the amplitude of the systolic $Ca^{2+}$ transient.[48, 50, 51]

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 phospholamban (PLN)[3, 8, 44, 49]. PKA mediated phosphorylation of the LTCC complex increases channel opening probability and the net result is an increase in $Ca^{2+}$ 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 [$Ca^{2+}$] since there is already sufficient LTCC mediated $Ca^{2+}$ entry under basal conditions to fully activate EC coupling[46]. However, the releasable SR $Ca^{2+}$stores are increased by sympathetic activity via the additional LTCC $Ca^{2+}$ influx, to produce graded increases in theSR $Ca^{2+}$ load. The SR $Ca^{2+}$ release and SR $Ca^{2+}$ loading aspects of $Ca^{2+}$ entry through the LTCC have been well known for decades[52, 53].

PLN is an inhibitor of SERCa and in the absence of sympathetic agonists it restrains SERCa $Ca^{2+}$ transport. PKA mediated PLN phosphorylation at serine 16 removes the inhibitory effect of PLN on SERCa, resulting in enhanced SR $Ca^{2+}$ uptake. β-adrenergic agonists therefore increase $Ca^{2+}$ entry and stimulate $Ca^{2+}$ uptake by the SR to grade the amount of releasable $Ca^{2+}$ stored in the SR. The well-established mechanism[49] for grading cardiac contractility is by sympathetic stimulation of $Ca^{2+}$ influx[4]and SERCa[54]to grade the releasable $Ca^{2+}$ 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 over 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 usedaRyRS2808A (with no PKA-mediated RyRS2808 phosphorylation) knock-in mouse kindly provided by the Valdivia laboratory[48].Our experiments clearly showed that the RyRS2808A heart (in-vivo and in-vitro) and isolated RyRS2808A myocytes respond normally to β-adrenergic agonists[51]. 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 reexplored this issue and published very 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 [$Ca^{2+}$] transients and contraction in RyRS2808A myocytes.Their conclusion was that eliminating the effect of RyRS2808 phosphorylation on $Ca^{2+}$ dependent opening of RyR abolishesβ-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 two groups is not easily explained.

One of the issues that was inadequately explored in the study from the Marks group[32] is thatβ-adrenergic effects on the LTCC and PLN should have been intact in the RyRS2808A mouse. Therefore, $Ca^{2+}$ influx and SR $Ca^{2+}$ loading should have increased with catecholamine stimulation. Unfortunately these critical parameters were never measured[32]. Where did this extra $Ca^{2+}$ influx go? It is well established that when $Ca^{2+}$ influx increases in cardiac myocytes there must be an increase in the counterbalancing $Ca^{2+}$ efflux, to maintain the new steady state[55]. This is established, fundamental, normal physiology. If increased $Ca^{2+}$ influx is not met with the required increase in $Ca^{2+}$effluxmyocytes will$Ca^{2+}$ overload and die. Our results clearly document that catecholamines increase LTCC mediated $Ca^{2+}$ entry and increase SR $Ca^{2+}$ load to increase SR $Ca^{2+}$ release in both normal and RyRS2808A myocytes. The resultant increase in the systolic $Ca^{2+}$ transient increases $Ca^{2+}$ 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 [$Ca^{2+}$] transient required to produce $Ca^{2+}$ flux balance in the RyRS2808A myocytes was not explained in the studies from the Marks laboratory[32]. There is no reason to believe that catecholamines failed to increase $Ca^{2+}$ influx through the LTCC and $Ca^{2+}$ uptake by the SR. It is unclear how was a new flux balance was achieve.? Since these critical issues were not adequately examined I assume that these RyRS2808A mice either do not have catecholamine dependent regulation of the LTCC or $Ca^{2+}$was transported out of myocytesvia a novel mechanisms that does not require an increase in the systolic $Ca^{2+}$ 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 believe that this single amino acid is essential for the moment-to-moment regulation of myocyte contractility[32]. So how does a RyRS2808A mouse, which requires phosphorylation at RyRS2808for 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 $Ca^{2+}$ regulatory proteins[51].However, we found no changes in the abundance or phosphorylation state of any relevant $Ca^{2+}$ regulatory protein in the RyR S2808A mouse51. In my opinion the loss of an essential $Ca^{2+}$ regulatory mechanism should have induced adaptive changes in other related $Ca^{2+}$ regulatory processes.I form this opinion based on results of others who have shown that when a critical protein involved in myocyte $Ca^{2+}$ regulation is eliminated or disrupted there are major adaptive changes in other components of $Ca^{2+}$ regulation[56, 57]. An example is when the major $Ca^{2+}$ efflux mechanism, the Na/Ca exchanger (NCX), is conditionally deleted (in most myocytes). This conditional Na/Ca exchanger deficient mouse adapts by significantly reducing the expression of the major $Ca^{2+}$ influx pathway, through the

LTCC[57]. The mouse with forced reduction in $Ca^{2+}$ efflux capacity immediately adapts by reducing $Ca^{2+}$ influx. Basal state function is fairly well preserved but with consequences, the animal is stress intolerant[57]. Our studies[50, 51] failed to show any alterations in the adrenergic regulation of $Ca^{2+}$ current, EC coupling, myocyte [$Ca^{2+}$] 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 group[32]misquoted aspects of our published work[51].The facts are that we used[51] a 10 fold lower (10 nM) isoproterenol concentration than used in their study[32] of isolated RyRS2808A hearts (100 nM).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 two studies[32, 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 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 two 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[58]. 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 if PKA-mediated hyperphosphorylation of RyRS2808 is singularly responsible for altered RyR function in the failing heart. WhileI believe 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[59]by oxidative stress pathways[60] 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[62] and CPVT[63].

The data published in support of the PKA-mediated RyRS2808 hypothesis[16, 18–20, 22–33, 64]shows 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 [$Ca^{2+}$] 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 appear 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 down regulation of adrenergic signaling cascades there is still persistent activation of β-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[65] 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 β-blockers RyRS2808 phosphorylation returns to normal, even though the heart failure state was still present (tachypacing model of CHF)[20]. Since β-blockers are standard of care for heart failure patients, 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 β-blockers and this normalized theirRyRS2808 phosphorylation state. My personal view is that pretty much 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 cautionbecause major changes in protein posttranslational 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 believe there is every reason to believe that RyRs in failing myocytes have significant posttranslational modifications including phosphorylation changes at S2808.

## Does RyRS2808 hyperphosphorylation cause FKBP12.6 to dissociate from RyR and reduce the [$Ca^{2+}$] 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 s-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 believe 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 has 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[68] 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, Calcineurin over expression, isoproterenol, chronic and severe TAC) and pre heart failure models (acute or mild TAC, angiotensin II, aldosterone, hypertrophy, ischemia reperfusion injury)(reviewed in[58]). 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 factorthat 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 suggests 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[30]. The idea that CaMKII is not activated in CHF induced by ischemic heart disease seems highly unlikely[58].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[58, 68–70] in ischemic heart failure has not been adequately explained and is inconsistent with published data[58].The notion that RyRS2808 phosphorylation is involved in RyR modifications in ischemic but not hypertrophic heart diseasealso does not fit with a host of previous studies[58,65].The abundance of existing data in the strongly supports the idea that CaMKII is pathologically activated(autophosphorylated and/or oxidized) in all forms of human heart failure and that CaMKII mediated phosphorylation of RyRS2814 should be present in ischemic cardiomyopathy[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[30,33]. 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[64].Given my laboratories long interest in the bases of contractility defects in human heart failure I felt compelled to see if my group could confirm the findings that RyRS2808A knock in mice are protected from cardiac dysfunction after MI. Dr. Hector Valdivia again was kind enough to share his mice[48] with us. He had already shown that RyRS2808A mice were not protected from pressure overload induced alterations in cardiac structure and function[48]. We then performed a complementary study in which RyRS2808A mice were subjected to MI[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[50].

The Wehrens lab has recently found no change in RyRS2808 phosphorylation in the failing human heart (any etiology) and has shown that RyRS2814A fails to protect mouse hearts after MI[65].As discussed above, our study shows that RyRS2808A also fails to protect the heart after MI[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 $Ca^{2+}$ 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[58]. The next step will be to determine if pharmacological agents that eliminate CHF induced RyR functional alterations make things better or worse. A parting thought is that maybe SR $Ca^{2+}$ leak is a good thing in the $Ca^{2+}$ stressed, failing heart? Maybe leak reduces the SR $Ca^{2+}$ overload that promotes myocyte death signaling or enhances arrhythmias? Time and unbiased, high quality science will tell.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Non-standard abbreviations and non-standard acronyms

| | |
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| **RyR** | Ryanodine Receptor (SR $Ca^{2+}$ release channel) |
| **RyRS2808** | Serine at amino acid 2808 on RyR |
| **RyRS2808A** | Alanine substitution for Serine at amino acid 2808 on RyR |
| **SR** | Sarcoplasmic Reticulum |
| **NCX** | Sodium-Calcium exchanger |
| **PLN** | Phospholamban |
| **PKA** | Protein Kinase A |
| **CaMKII** | $Ca^{2+}$-Calmodulin Kinase II |
| **CHF** | Congestive Heart Failure |

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|---|---|
| **SERCa** | SR $Ca^{2+}$ ATPase |
| **TAC** | Thoracic aortic constriction |
| **LTCC** | L-type $Ca^{2+}$ channel |
| **CPVT** | Catecholaminergic Polymorphic Ventricular Tachycardia |
| **FKBP12.6** | FK Binding Protein 12.6 |

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