Role of RyR2 Phosphorylation in Heart Failure and Arrhythmias

Controversies Around Ryanodine Receptor Phosphorylation in Cardiac Disease

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Abstract: Cardiac ryanodine receptor type 2 plays a key role in excitation–contraction coupling. The ryanodine receptor type 2 channel protein is modulated by various post-translational modifications, including phosphorylation by protein kinase A and Ca²⁺/calmodulin protein kinase II. Despite extensive research in this area, the functional effects of ryanodine receptor type 2 phosphorylation remain disputed. In particular, the potential involvement of increased ryanodine receptor type 2 phosphorylation in the pathogenesis of heart failure and arrhythmias remains a controversial area, which is discussed in this review article. (Circ Res. 2014;114:1311-1319.)

Key Words: atrial fibrillation ■ heart failure ■ phosphorylation ■ ryanodine receptor type 2

This review focuses on recent controversies surrounding studies addressing the consequences of ryanodine receptor type 2 (RyR2) phosphorylation in heart. RyRs serve as Ca²⁺-release channels on endo/sarcoplasmic reticulum (SR) of excitable tissues, including neurons, skeletal, and cardiac muscle. During the past 20 years, >900 publications have focused on the role of RyR phosphorylation (Figure 1). Circulation Research has published the largest share of these articles, >80 in total, including some of the most controversial work on RyR2 phosphorylation in the pathogenesis of cardiac disease. Several recent reviews have summarized major studies in the field and already described some of the inconsistent and partly opposing conclusions thereof. Here, we will try to delineate which concepts can be considered as mostly accepted, which require further in-depth investigations, and will discuss major unresolved controversies in the field.

Counterpoint, see p 1320
Response by Houser, see p 1319

RyR2 Phosphorylation and Excitation–Contraction Coupling in Normal Hearts

RyR2 is the major SR Ca²⁺-release channel involved in excitation–contraction coupling (Figure 2), the process by which an electric depolarizing impulse is transduced into a cardiac contraction. The amount of Ca²⁺ released from the SR via RyR2 largely determines the Ca²⁺-transient amplitude, which correlates with the strength of systolic contraction. The RyR2 channel consists of 4 pore-forming subunits, which associate with numerous accessory proteins including FK506-binding protein-12.6 (FKBP12.6), calmodulin, calsequestrin-2, junctin, triadin, and junctophilin-2, all of which can regulate channel gating. In addition, RyR2 is regulated at the post-translational level by S-nitrosylation, oxidation, and protein phosphorylation.

RyR2 channels contain several phosphorylation sites. The degree of steady-state phosphorylation of each site depends on a dynamic balance between multiple protein kinases and phosphatases, allowing precise control of RyR2 phosphorylation and, consequently, channel activity. Alterations in RyR2 phosphorylation play a critical role in various cardiac diseases, including heart failure (HF) as well as atrial and ventricular arrhythmias. Most studies to date have only focused on the effects of protein kinase A (PKA) and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII)–mediated RyR2 phosphorylation, although it is likely that other serine/threonine protein kinases can also phosphorylate RyR2.
Serine-2808 (S2808, or S2809 in some species) was the first RyR2 residue identified as a phosphorylation site and is thought to be the primary target of PKA phosphorylation. Addition of purified PKA to a single RyR2 channel studied in the planar lipid-bilayer system increased RyR2 open probability (Po). The increased RyR2-Po was prevented by mutating S2808 to alanine (S2808A), rendering the channel unphosphorylatable. Conversely, the phosphomimic mutation S2808 to aspartate (S2808D) increased RyR2-Po in some, but not all, studies. Additional studies in ventricular cardiomyocytes from S2808A mice showed that the RyR2 phosphorylation level alters channel activity in intact cardiomyocytes, as evidenced by changes in Ca\textsuperscript{2+}-transient amplitudes and Ca\textsuperscript{2+}-spark frequencies, in some, but not other, studies. Additional studies were performed by treating wild-type ventricular cardiomyocytes with PKA inhibitors. However, it is impossible to attribute the observed changes (or lack thereof) to phosphorylation of a specific RyR2 residue, because all PKA phosphorylation sites and other PKA target proteins will be simultaneously modified. Therefore, studies using knock-in mouse models of missense mutations of specific phosphorylation sites remain invaluable to study specific sites, although they also have intrinsic limitations (see below). Even using similar S2808A knock-in mouse lines, opposing findings have been reported for the functional effects of S2808 phosphorylation. For example, Shan et al reported blunted heart rate and contractile responses to isoproterenol and a blunted enhancement of Ca\textsuperscript{2+}-transient amplitude and fractional shortening in S2808A mice. In contrast, MacDonnell et al showed unaltered chronotrophic and inotropic responses to isoproterenol in vivo and in vitro. It is possible that there is a threshold below which PKA phosphorylation of RyR2 (at S2808) does not cause a physiological effect, but that does not seem to be a sufficient explanation for these clear differences. Nevertheless, it is intriguing why well-established and leading laboratories in the field using similar mutant recombinant channels and knock-in mouse models arrive at opposite conclusions regarding the physiological effects of PKA-dependent RyR2 phosphorylation, particularly at the S2808 site.

Several factors might contribute to these discrepancies, including subtle differences in the animal models, reagents, experimental procedures, and data interpretation (summarized in the Table). Furthermore, new findings continue to refine our understanding of signaling pathways and RyR2 regulation. For example, initially PKA phosphorylation of S2808 was reported to reduce FKBP12.6 binding. However, S2808D knock-in mice with constitutively phosphorylated RyR2 channels exhibited normal FKBP12.6 binding, at least at a young age. We will not discuss the controversies related to FKBP12.6 in relation to RyR2 regulation and phosphorylation because of space limitations, but will refer to prior excellent review articles. Recent studies revealed that oxidation and S-nitrosylation, together with RyR2 phosphorylation at S2808, are required to dissociate FKBP12.6 from RyR2 and to increase RyR2-Po. At present, the exact mechanisms underlying the synergy between these different post-translational RyR2 modifications remain unknown and require follow-up studies.

Serine-2814 (S2814, in some species S2815) was the second RyR2 residue identified as a primary CaMKII target. Careful stoichiometric analysis revealed that in vitro CaMKII phosphorylation could partially phosphorylate a second still- unidentified residue. One study claimed that CaMKII can phosphorylate 3 to 4 sites, but these conclusions were not based on stoichiometric studies using quantifications of both total and phosphorylated RyR2. Claims in the literature including some review articles that there are 3 to 4 or even more phosphorylation residues per RyR2 monomer are not based on credible data and will not help to resolve this controversy. However, there is a broad consensus that CaMKII-mediated RyR2 phosphorylation increases RyR2-Po, both at the single-channel level in bilayers and in ventricular cardiomyocytes as evidenced by increased Ca\textsuperscript{2+} spark frequency. Mutation S2814A prevents most CaMKII effects on RyR2, suggesting that S2814 is the major, but probably not the exclusive, RyR2 residue subject to CaMKII phosphorylation. These findings are consistent with studies in CaMKII-knockout and transgenic animals revealing altered RyR2 phosphorylation at S2814 and altered Ca\textsuperscript{2+} spark frequency depending on global CaMKII levels.

Some data suggest a cross-talk between the S2808 and S2814 phosphorylation sites. For example, enhanced S2814 phosphorylation was observed in S2808A knock-in mice, suggesting that the phosphorylation state of 1 residue might affect
the likelihood that neighboring residues are phosphorylated, particularly after adrenergic stimulation. Alternatively, this may be an artifact attributable to changes in the epitope for the phospho-specific antibodies. Therefore, it is important that all known RyR2 phosphorylation sites are monitored when studying (patho)physiological mechanisms using RyR2 knock-in mouse models.

Some years ago, serine-2030 (S2030, or S2031 in some species) was described as a third functional RyR2 phosphorylation site. PKA phosphorylation of this site might enhance RyR2 sensitivity to luminal (intra-SR) Ca\(^{2+}\). Other studies, however, have failed to demonstrate a measurable functional effect of this phosphorylation site, and its physiological significance remains to be determined in vivo. Overall, it would be of great interest to identify other RyR2 phosphorylation sites that are regulated under physiological conditions or perhaps altered in cardiac diseases, to determine the exact basal level of RyR2 phosphorylation at steady state for each individual residue, to identify the specific kinases and their counterbalancing phosphatases that dynamically phosphorylate/dephosphorylate each respective residue, and to delineate the precise allosteric mechanisms underlying the synergistic effects of concomitant phosphorylation at multiple sites, along with other post-translational modifications of RyR2 subunits.

Dephosphorylation of RyR2 is mediated by protein phosphatase type 1 (PP1), which is targeted to the complex by the regulatory subunit spinophilin, and PP2A, which is targeted through its regulatory subunit PR130. PP2A may be also targeted to RyR2 through B56a binding to ankyrin-B, which also interacts with RyR2, or through the muscle-specific A kinase–anchoring protein, which binds both RyR2 and PP2A-B56a. Steady-state phosphorylation of individual RyR2 phosphorylation sites is regulated in a highly complex and dynamic manner, which should be seriously considered when quantifying the degree of phosphorylation and studying its consequences for cardiac (dys)function. Several studies have demonstrated that PP-mediated dephosphorylation of RyR2 decreased channel open probability, but other studies have reported the opposite. Moreover, it was shown that PP increases RyR2 leakiness in cells expressing wild-type, but not S2808A mutant, RyR2 with the disabled PKA phosphorylation site. Thus, the PP-mediated regulation of RyR2 remains controversial at this time.

**Figure 2. Schematic representation of the key determinants of excitation–contraction coupling in cardiomyocytes.** Physiologically, Ca\(^{2+}\) entry through L-type Ca\(^{2+}\) channels (\(I_{\text{Ca,L}}\)) triggers sarcoplasmic reticulum (SR) Ca\(^{2+}\) release through ryanodine receptor type 2 (RyR2). The systolic Ca\(^{2+}\) transient activates myofilaments, initiating contraction. Diastolic relaxation occurs when Ca\(^{2+}\) is transported into SR via SERCA2a and out of the myocyte via Na\(^{+}\)/Ca\(^{2+}\) exchanger 1 (NCX1). In heart failure (HF) and atrial fibrillation (AF), altered RyR2 phosphorylation increases SR Ca\(^{2+}\) leak, promotes Ca\(^{2+}\)-dependent remodeling, and impairs contractility. Spontaneous SR Ca\(^{2+}\)-release events (SCaEs) promote delayed afterdepolarizations (DADs) and triggered activity. Inset shows RyR2 macromolecular complex with accessory proteins, protein kinases, and phosphatases (and their respective anchoring proteins) that control phosphorylation levels. Protein kinase A (PKA) and Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII)–dependent phosphorylation sites are indicated with blue and green P symbols, respectively. Ank-B indicates ankyrin-B; CSQ2, calsequestrin-2; FKBP12.6, FK506-binding protein-12.6; mAKAP, muscle-specific A kinase–anchoring protein; MyBP-C, myosin binding protein C; PLB, phospholamban; PP1, protein phosphatase type 1; and \(\beta\)-AR, \(\beta\)-adrenergic receptor.
Role of Altered RyR2 Phosphorylation in HF

Enhanced RyR2-mediated SR Ca\(^{2+}\) leak has been observed in patients with HF and various animal models.\(^{13,14,15,16}\) It is likely that diastolic SR Ca\(^{2+}\) leak, together with reduced sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA2a) function and enhanced Na\(^{+}/\)Ca\(^{2+}\) exchanger type 1 function, contributes to the depletion of SR Ca\(^{2+}\) content in HF.\(^5\) The causal role of RyR2 phosphorylation in HF pathogenesis, however, remains highly controversial because different groups have reported contradictory findings using similar approaches and animal models.\(^{15,16,22}\) In addition, RyR2 is regulated by various additional post-translational modifications, and the potential synergistic or antithetic effects with phosphorylation remain largely unexplored.\(^{44-66}\)

The first article demonstrating altered RyR2 phosphorylation in patients with HF was published in 2000 by Dr Marks’ group.\(^{14,15,17}\) These authors\(^{14}\) postulated that the hyperadrenergic state in HF increases PKA-mediated S2808 phosphorylation, reduces FKBP12.6 binding to RyR2, and enhances RyR2-Po. Several (but not all) subsequent articles validated the increased S2808 phosphorylation in HF in various species, as previously summarized.\(^{13,33}\) In some cases, different groups used similar mutant mice (S2808A knock in) and seemingly similar experimental models, yet obtained opposite results.\(^4\) For example, the Marks laboratory demonstrated in S2808A knock-in mice that S2808 phosphorylation is a critical mediator of progressive cardiac failure after experimental myocardial infarction (MI).\(^15\) In contrast, the Houser laboratory showed no protective effects in S2808A mice (generated independently by their group), despite a significant increase in S2808 phosphorylation after MI.\(^{22}\) Once again, numerous factors might contribute to the discrepant results (Table). Complementary studies in knock-in mice of the constitutively hyperphosphorylated S2808 site (S2808D mice) revealed the spontaneous development of an age-dependent cardiomyopathy, which might support a role for this site in some types of HF development.\(^17\)

It is important to consider several general concepts when comparing studies in this field. For example, because cardiac remodeling is likely heterogeneous throughout the heart, RyR2 phosphorylation levels might vary among different regions.\(^{57}\) Furthermore, some studies suggest that the pathogenesis of HF might affect the relative phosphorylation level of different RyR2 sites.\(^{10,32,33}\) It is also unclear whether RyR2 phosphorylation sites are differentially phosphorylated during different stages of HF or in the context of different causes of the disease.\(^68\) Recent studies on human hearts revealed that both S2808 and S2814 are hyperphosphorylated during compensated cardiac hypertrophy, whereas only S2814 remained phosphorylated in patients with end-stage HF.\(^{68}\) At present, it is unclear how common risk factors such as metabolic syndrome, diabetes mellitus, and ischemia (ie, oxidative stress) might impact RyR2 phosphorylation. Additional studies in larger patient populations and large animal models with well-controlled conditions are required to resolve such important questions.

There is now strong evidence that abnormal CaMKII phosphorylation of RyR2 can contribute to contractile dysfunction in HF.\(^{33,48,69}\) First, several studies showed that CaMKII hyperactivity can cause HF, for example, in CaMKII-transgenic mice,\(^{70}\) whereas mice overexpressing a CaMKII inhibitor or deficient in CaMKII-δ are protected from developing HF.\(^{55,71}\) Second, RyR2 has been identified as a major downstream target of CaMKII involved in abnormal SR Ca\(^{2+}\) leak and contractile dysfunction in HF.\(^{69,72}\) Our studies of a limited number of human failing hearts revealed increased RyR2 phosphorylation at...
S2814 in nonischemic dilated cardiomyopathy but not in ischemic cardiomyopathy. These findings suggest that CaMKII activation might depend on the type of HF.

Mouse HF models demonstrated that S2814 phosphorylation was increased in mice subjected to transverse aortic constriction but not after MI. S2814 ablation in S2814A mice prevented progression of cardiomyopathy to severe HF in these mice, whereas no beneficial effect was noted in S2814A mice subjected to MI. Moreover, constitutive S2814 phosphorylation in S2814D mice caused spontaneous late-onset HF. These data suggest that S2814 phosphorylation might be involved in adverse cardiac remodeling in dilated cardiomyopathy, although additional studies are required to confirm this. These studies do not exclude the possibility of additional CaMKII phosphorylation sites on RyR2 playing a role in dilated cardiomyopathy or other types of HF.

Some studies have demonstrated CaMKII activation in failing hearts after MI. However, consistent with the lack of S2814 hyperphosphorylation after MI in mice, it was not too surprising that S2814A knock-in mice were not protected from HF progression after MI. These findings are also in agreement with a study from another laboratory showing a lack of protection from MI-induced HF in S2814A mice.

Role of RyR2 Phosphorylation in Ventricular Arrhythmogenesis

Many patients with HF die suddenly because of ventricular arrhythmias, many of which are thought to be initiated by focal triggered activity, involving spontaneous diastolic SR Ca2+-release events (SCaEs) via RyR2. Sudden increases in SR Ca2+ leak can activate a potentially arrhythmogenic depolarizing inward Na+/Ca2+ exchange current, which can cause delayed afterdepolarizations and trigger ventricular arrhythmias. CaMKII, which is upregulated and more active in HF, has been shown to promote SR Ca2+ leak associated with triggered arrhythmias.69,76,77

Studies in S2814D mice with constitutively phosphorylated RyR2 revealed an increased risk for ventricular arrhythmias, even in the absence of structural heart disease. Interestingly, although S2814D mice show more spontaneous Ca2+ sparks, ectopic activity was not observed under resting conditions, suggesting that a combination of increased SR Ca2+ load (during fast pacing/β-adrenergic stimulation) is required to initiate arrhythmias. It remains to be studied whether concomitant S2808 and S2814 hyperphosphorylation promotes severe arrhythmogenic SR Ca2+ leak, even in the absence of β-adrenergic stimulation. In addition, S2814 phosphorylation might be a key mechanism for triggered arrhythmias in HF, at least following transverse aortic constriction in mice. Thus, it is mostly accepted that CaMKII-mediated RyR2 phosphorylation leads to SR Ca2+ leak associated with arrhythmias, but the potential role of S2808 phosphorylation in arrhythmogenic SCaEs remains uncertain. Finally, in patients with HF, several mechanisms likely conspire to promote triggered activity and ventricular tachycardia, including enhanced phosphorylation, oxidation, and S-nitrosylation of RyR2. The mechanisms underlying such synergistic effects are mostly understudied and should be addressed in future studies.

RyR2 phosphorylation is also thought to participate in the pathogenesis of catecholaminergic polymorphic ventricular tachycardia (CPVT), an inherited condition characterized by exercise- or stress-induced arrhythmias, syncope, and sudden cardiac death. More than half of the CPVT cases are caused by autosomal-dominant missense mutations in RyR2. Although >150 different RyR2 mutations have been reported, none seem to occur within known phosphorylation motifs. Clinically, potentially lethal arrhythmias occur predominantly after strenuous exercise or emotional stress, presumably in association with increased adrenergic activity. At the cellular level, enhanced adrenergic activity activates PKA and CaMKII, leading to enhanced RyR2 phosphorylation.77,82 Most experimental studies agree that RyR2 phosphorylation can potentiate SR Ca2+ leak, which is associated with ectopic activity and arrhythmias in CPVT. However, some issues remain controversial, as discussed below.

We will focus only on the role of RyR2 phosphorylation in CPVT and refer the reader to prior reviews about other aspects of CPVT including the potential role of reduced FKBP12.6 binding and domain unzipping as mechanisms of RyR2 destabilization.

It is somewhat controversial whether CPVT-associated RyR2 mutations cause channel dysfunction in the absence of adrenergic stimulation and channel phosphorylation. Several studies have shown that RyR2 phosphorylation uncovers latent single-channel dysfunction of mutant RyR2. Other studies have shown that only SR Ca2+ overload, without activation of PKA, can unmask the gain-of-function phenotype of CPVT-mutant RyR2 variants. However, other studies demonstrate a baseline gain-of-function89 or even a rare loss-of-function phenotype of CPVT-mutant RyR2 channels. These differences might result from different experimental approaches used or may reflect differences in phenotype severity depending on the specific affected residue. For example, mutation V2475F seems to cause a particularly severe phenotype because homozygous knock-in mice are not viable, unlike other mutant gain-of-function RyR2 strains. Interestingly, the neighboring mutation R2474S also causes a rather severe arrhythmia phenotype, compared with other RyR2 mutants (L433P, N2386I) in side-by-side studies. Most studies show that the CPVT-linked mutations do not alter baseline RyR2 phosphorylation at S2808 and S2814. However, recombinant RyR2 channels with the V2475F mutation exhibited significantly increased S2808 and S2030 phosphorylation levels at baseline that were even more pronounced after exposure to the PKA catalytic subunit. However, the composition of recombinant RyR2 is likely different from native heart channels, so future experiments will need to confirm whether CPVT mutations alter basal RyR2 phosphorylation levels.

Role of RyR2 Phosphorylation in Atrial Arrhythmogenesis

Atrial fibrillation (AF) is partially characterized by Ca2+-handling abnormalities. Initial work showed that the incidence of spontaneous Ca2+-release events (SCaEs, which include Ca2+ sparks and Ca2+ waves) is increased in cardiomyocytes from patients with chronic (persistent) AF (cAF), pointing primarily to RyR2 dysfunction. Emerging evidence indicates that abnormal RyR2 phosphorylation indeed plays a prominent role in AF pathogenesis. RyR2 is hyperphosphorylated at S2808 in both patients with cAF and dogs with sustained AF because of atrial tachycardia remodeling. Increased S2808 phosphorylation of RyR2 in patients with cAF was surprising because cytosolic PP1 and PP2A activities are increased, which would be
expected to reduce RyR2 phosphorylation levels.\textsuperscript{97} Despite the larger SR Ca\textsuperscript{2+} leak, SR Ca\textsuperscript{2+} load is unaltered in cAF\textsuperscript{98} possibly because of increased phospholamban phosphorylation.\textsuperscript{97}

In dogs, the increase in S2808 phosphorylation of RyR2 was associated with a stronger dissociation of FKBP12.6 subunit from RyR2 channels.\textsuperscript{89} Mice lacking FKBP12.6 showed larger SR Ca\textsuperscript{2+} leak and more SCaEs and triggered activity, along with an increased susceptibility to burst pacing–induced AF, validating the causal role of reduced FKBP12.6 levels for RyR2 dysfunction.\textsuperscript{41,42,99} Similar results were obtained in mice with the E169K mutation in junctophilin-2, which reduced its interaction with RyR2, suggesting an important RyR2-stabilizing role also for junctophilin-2.\textsuperscript{2,100} Gain-of-function mutations in RyR2 predispose patients to CPVT and AF, and mice with these CPVT mutations show RyR2 dysregulation and burst pacing–induced AF.\textsuperscript{41,93,101} The RyR2–FKBP12.6 binding stabilizing compound S107 prevents AF initiation in CPVT mice, pointing to a role of FKBP12.6 for RyR2 dysfunction in CPVT-related AF.\textsuperscript{93}

The individual contribution of S2808 phosphorylation to RyR2 dysregulation and SR Ca\textsuperscript{2+} leak in patients with cAF is uncertain. Although RyR2 hyperphosphorylation at S2808 seems to be a consistent finding in patients with cAF,\textsuperscript{41,96,98,102} pharmacological PKA inhibition does not affect the increased RyR2-Po and SR Ca\textsuperscript{2+} leak in atrial myocytes from patients with cAF.\textsuperscript{98} Although these data intuitively question an important role of S2808 phosphorylation, the apparent lack of S2808 contribution might be attributable to the atrial tachycardia-induced permanent increase in CaMKII activity and S2814 hyperphosphorylation, which may mask the impact of S2808 phosphorylation for atrial function. In addition, the S2814 hyperphosphorylation might cause a stronger conformational change of RyR2 resulting in a larger increase in RyR2-Po. Extensive additional work is needed to address these interesting possibilities.

Several articles have demonstrated that increased CaMKII-dependent RyR2 phosphorylation at S2814 is the major cause of SR Ca\textsuperscript{2+} leak and SCaEs in patients with cAF.\textsuperscript{41,43,98,102} CaMKII activity may increase as a result of faster atrial rate, which promotes its autophosphorylation,\textsuperscript{10} or from oxidation of methionines 281/282, coupling AF-related oxidative stress to proarrhythmic Ca\textsuperscript{2+} handling.\textsuperscript{2,99} In addition, Thr35 hyperphosphorylation of I-1 in cAF is expected to reduce PP1 activity within the RyR2 complex,\textsuperscript{9,10} also increasing S2814 phosphorylation. In goats with sustained AF, CaMKII-dependent RyR2 phosphorylation is increased, likely causing SR Ca\textsuperscript{2+} leak, potentially contributing to the reduced SR Ca\textsuperscript{2+} load and decreased atrial contractility associated with AF.\textsuperscript{94,104}

Several mouse models have validated the causal role of CaMKII- and S2814-mediated RyR2 dysfunction, increased SR Ca\textsuperscript{2+} leak, and SCaEs in AF initiation.\textsuperscript{41,43,98,102} Pharmacological CaMKII inhibition and genetic inhibition of CaMKII-dependent S2814 phosphorylation prevent AF initiation in FKBP12.6-knockout mice, further supporting a critical role for S2814-mediated RyR2 dysfunction in AF.\textsuperscript{41,99} Mice with cardiac-restricted overexpression of a repressor form of the cAMP-response element modulator (CREM) develop a complex cardiac phenotype including spontaneous-onset AF.\textsuperscript{105,106} CREM transgenic mice exhibit changed atrial structure and hypertrophy, along with altered conduction and Ca\textsuperscript{2+}-handling abnormalities including increased incidence of SCaEs and augmented SR Ca\textsuperscript{2+} leak.\textsuperscript{106} This mouse model supports a critical role for S2814 phosphorylation–dependent RyR2 dysfunction in spontaneous AF.\textsuperscript{106} CaMKII-dependent RyR2 hyperphosphorylation is likely an early event in atrial CREM transgenic mice dysfunction, because CREM transgenic mice crossed with RyR2-S2814A mice resistant to CaMKII-dependent RyR2 hyperphosphorylation are protected from spontaneous AF.\textsuperscript{106} Overall, these studies support a major role for S2814 phosphorylation of RyR2 in atrial dysfunction and arrhythmogenesis in persistent AF.

Interestingly, patients with paroxysmal AF do not exhibit changes in RyR2 phosphorylation at S2808 and S2814, despite increased SCaEs incidence and triggered activity.\textsuperscript{44} The underlying molecular substrate involves increased SR Ca\textsuperscript{2+} load and RyR2 dysregulation independent of RyR2 phosphorylation. The increased SR Ca\textsuperscript{2+} load is attributable to PKA-dependent phospholamban hyperphosphorylation, relieving phospholamban inhibition of SERCA2a and increasing SR Ca\textsuperscript{2+} uptake.\textsuperscript{44} RyR2 dysregulation involves increased protein expression and higher RyR2-Po, resulting in larger likelihood and amplitude of SCaEs. A relative deficiency of junctophilin-2, resulting from increased RyR2 but unaltered junctophilin-2 expression, might explain RyR2 dysfunction in patients with paroxysmal AF.\textsuperscript{44,100}

### Conclusions

Many studies have demonstrated that phosphorylation is an important mechanism by which RyR2-mediated SR Ca\textsuperscript{2+} release is fine-tuned within cardiomyocytes.\textsuperscript{3} Although our knowledge in this field is still evolving and certain concepts remain controversial because of disagreements among certain studies, it is evident that abnormal PKA and particularly CaMKII phosphorylation of RyR2 may contribute to the pathogenesis of HF and atrial and ventricular arrhythmias. The fact that not all studies agree is not uncommon in science and actually helps to advance the field as a whole. It is important that there will be unrestricted exchange of reagents such as plasmids, antibodies, and knock-in mouse models among laboratories to enable external confirmation of published results. As new scientific insights and technologies become available, some of the outstanding questions will undoubtedly be resolved. In the meantime, it is important that we all focus on the generation of new insights rather than on the perpetuation of (perceived) controversies, especially if our conclusions are based on imperfect experimental approaches. Finally, it needs to be emphasized that advancing our understanding of RyR2 regulation and dysfunction in the context of cardiac diseases is of utmost importance because RyR2 represents a unique and promising therapeutic target for HF and arrhythmias.\textsuperscript{107,108}

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Disclosures

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These 2 controversies address a series of publications beginning in 2000 showing that protein kinase A (PKA)–mediated phosphorylation of a single serine (RyR2S2808) on the Ca"+-release channel (ryanodine receptor [RyR]) is essential for normal adennergic regulation of cardiac contractility and that hyperphosphorylation of this same amino acid is responsible for the sarcoplasmic reticulum–dependent contractility defects that provoke heart failure and arrhythmias in disease. Many in the field have been unable to confirm critical aspects of the original findings, and this has led to controversies regarding their soundness. Based on the 2 controversial articles appearing in Circulation Research, there seem to be some areas of agreement and some topics that remain unresolved and still deserve study. There is agreement that many aspects of the original PKA-RyR2S2808 phosphorylation hypothesis have not been confirmed. As an example, the initial studies showed that there was no role for Ca"+-independent SR Ca"+ leak in heart failure. There is also agreement that RyR regulation in health and disease is complex and involves multiple post-translational modifications rather than just 1 PKA phosphorylation site. There is agreement that RyR function is altered in disease and contributes to reduced contractility reserve and arrhythmias. There is agreement that strategies to re-establish normal RyR function, as a potential therapeutic for contractility defects and arrhythmias, should continue to be tested.

There are also areas where data sets differ and additional work is needed. As an example, the data suggesting that heart failure of different causes (ischemic or pressure overload–induced heart failure) uniquely involves either PKA or Ca"+-calmodulin-dependent protein kinase II–mediated dysregulation of RyR do not fit with the majority of data in the literature and deserve to be tested further. Systolic heart failure is a syndrome in which the pump function of the heart is reduced, and this always results in a hyperadrenergic state with Ca"+ stress. If this new idea is correct, then patients with ischemic or pressure overload–induced heart failure should respond differently to standard-of-care therapies such as ß-adrenergic receptor antagonists, which to my knowledge is not the case.

In summary, I think that controversies are good for any field. They stimulate new work and often lead to important new ideas. They induce passionate debate and sometimes they are even resolved. I applaud Dr Wehrens for engaging in this debate. I was only able to contribute to the RyR-PKA hyperphosphorylation controversy because of the generosity of Dr Hector Valdivia, who freely shared his phosphorylation-deficient RyR2S2808A mouse with my group. Without his willingness to share his genetically modified mice, my group would not have been able to contribute to this field. I want to take this opportunity to thank him for his generosity.
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