Ryanodine Receptor S2808 Phosphorylation in Heart Failure
Smoking Gun or Red Herring

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In this issue of Circulation Research, Zhang et al. provide direct and clear evidence that phosphorylation of S2808 in the cardiac ryanodine receptors (RyR2) has no impact on either normal cardiac function, sympathetic stimulation of the heart, or progression of heart failure (HF) after myocardial infarction (MI). They used mice in which the S2808 site was rendered nonphosphorylatable in S2808A knock-in mice, and the results directly contradict results published by the group of A.R. Marks.2–4 This has been a highly controversial area.

This new study compels me to assess this pathway in a balanced way, but recognizing that my opinions are influenced by work that my group has done in this area. My goal here is not to microdissect each controversial piece of data in dozens of relevant papers (see recent reviews and tables5,6), but rather to provide my informed opinion about key aspects of this issue in a balanced manner. Let us first consider the model that importantly raised the issue of SR Ca leak as a potential pathophysiological cardiac effect in HF. Although most investigators in this field embraced this idea initially and performed studies to clarify key aspects of the working model, many strong research groups in this field around the world have been unable to confirm numerous aspects of this working hypothesis.5,6 The Marks group has rigidly adhered to their initial detailed mechanism, and have consistently provided high-quality results that fully support their model. They have also largely dismissed the validity of many published results that have challenged their dogma. This situation has created tremendous controversy and tension in this field, and frustration in the broader field that things remain unresolved.

The present paper and 2 previous studies by the Houser and Valdivia groups have used the same strategy (RyR2 S2808A knock-in mice, although not the same mouse line)1,10,11 and very similar procedures as used by Marks et al.2,3 to test whether S2808 phosphorylation is critical in the sympathetic fight-or-flight effects on the heart and cardiac dysfunction induced by stress. These groups get diametrically opposite results. The Marks group finds that S2808A severely limits (a) the inotropic response to β-adrenergic receptor (β-AR) agonists, (b) the enhanced RyR2 gating (that can be arrhythmogenic in HF), and (c) the cardiac dysfunction in postmyocardial ischemia (MI) hearts. The Houser–Valdivia team finds that S2808A mice are functionally indistinguishable from WT mice with respect to these same parameters, arguing that this highly touted site is functionally irrelevant for either the fight-or-flight response or post-MI cardiac function. Why the discrepancy? Because mouse phenotype is complex and the mice are not identical, one can always blame the differences on that (ie, the Valdivia–Houser mouse may have undergone adaptive changes that limit the difference versus wild-type). Obviously, the best way to clarify that would be for mice exhibiting the difference to be shared with other investigators. It is a shame that that has not happened since the initial 2006 paper.2

At a more fundamental mechanistic level this field has been complicated by numerous challenges to many of the key tenets of the Marks group dogma, but without satisfactory resolution. Let’s consider some of these steps.

Is the RyR Hyperphosphorylated in HF?

Many but not all groups find that the basal state of RyR2 phosphorylation at S2808 (or S2809 depending on species) is already rather high (eg, >50% of maximum). Although this may limit the potential increases in phosphorylation at that site, several groups (including us12) find that S2808 phosphorylation is increased in HF. However, this is already a point of contention, in which several experienced groups have failed to detect an increase in S2808 phosphorylation in...
HF. There is more consistent observation among groups that RyR2 phosphorylation is increased in HF at the nearby CaMKII site on RyR2 S2814, but that is not unanimous either. A third RyR2 PKA phosphorylation site (S2031) has been identified and reported to be more phosphorylated in HF, but has been studied in less detail. Most investigators would probably agree that in HF myocytes, phosphatase activity is increased globally but also that less phosphatase is associated with the RyR2. This would lead to a relative enhancement of RyR2 phosphorylation in HF (eg, versus reduced phospholamban phosphorylation) at both PKA and CaMKII sites. That, coupled with the hyperadrenergic state in HF, may combine to increase RyR2 phosphorylation. We often talk about S2808 and S2814 as if they are exclusive PKA and CaMKII targets, respectively, but that is probably not absolute (and other kinases may also phosphorylate RyR2). In conclusion, I think that yes, RyR2 phosphorylation is increased in HF at the nearby CaMKII site on RyR2 S2814, but that is not unanimous either. A third RyR2 PKA phosphorylation site (S2031) has been identified and reported to be more phosphorylated in HF, but has been studied in less detail. Most investigators would probably agree that in HF myocytes, phosphatase activity is increased globally but also that less phosphatase is associated with the RyR2. This would lead to a relative enhancement of RyR2 phosphorylation in HF (eg, versus reduced phospholamban phosphorylation) at both PKA and CaMKII sites. That, coupled with the hyperadrenergic state in HF, may combine to increase RyR2 phosphorylation. We often talk about S2808 and S2814 as if they are exclusive PKA and CaMKII targets, respectively, but that is probably not absolute (and other kinases may also phosphorylate RyR2).

**Does PKA-Dependent RyR Phosphorylation Alter RyR2 Function?**

For CaMKII, there is fairly good agreement (but again not universal) that CaMKII-dependent RyR2 phosphorylation increases RyR2 opening using a wide array of conditions and in different labs. For PKA, there is much more controversy about whether and how it alters RyR2 gating. Many groups, including ours, have found absolutely no effect of PKA-dependent RyR2 phosphorylation on diastolic SR Ca release (Ca sparks, waves, or overall SR Ca leak) when one controls for SR Ca load or in isolated lipid bilayer recording (including phosphomimetic S2808D channels). Valdivia et al found that PKA slightly depressed steady state RyR2 open probability (which is analogous to a slight reduction in diastolic SR Ca leak), but they also found that PKA increased the acute response to an instantaneous rise in local [Ca] (analogous to Ca current trigger during E-C coupling). The former point jibes with results above showing that PKA does not enhance SR Ca leak, and perfectly fits with work showing that PKA raises slightly the SR Ca threshold for spontaneous Ca wave initiation. The latter effect also matches our carefully controlled voltage clamped study in which PKA activation enhanced the rate of rise and termination of SR Ca release in response to a given Ca current trigger (and SR Ca load), but did not alter the amount of Ca release. I think that PKA phosphorylation of RyR2 may speed up the Ca-release process, but specifically without sensitizing to diastolic [Ca]. My conclusion is that PKA-dependent RyR phosphorylation in cardiac myocytes does not increase diastolic SR Ca release. That of course could make the PKA phosphorylation level at S2808 functionally irrelevant, at least with respect to diastolic SR Ca release.

**Does FKBP12.6 Dissociation From RyR2 in HF or on Phosphorylation by PKA?**

This central point of the Marks et al hypothesis is well supported by all of their work, but has limited support from other groups who have carefully assessed this. For example, we directly measured FKBP12.6 affinity, on-rates and off-rates in 3 different ways in the cardiac myocyte setting. Although FKBP12.6 affinity is very high for RyR2 in the cell (Kd~1 nmol/L), there was absolutely no effect of PKA on FKBP12.6 binding kinetics or affinity. Moreover, we found that in rat and mouse myocytes there is only enough FKBP12.6 present to bind to a small fraction of the RyR2 monomers, so most myocyte RyRs already lack FKBP12.6 (the supposed HF-altered state). I am convinced that PKA-dependent phosphorylation of RyR2 by itself does not alter FKBP12.6 binding.

**Does FKBP12.6 Dissociate From RyR2 Alter RyR2 Function?**

Marks et al hypothesize that FKBP12.6 stabilizes RyR2 gating and prevents long-lasting subconductance states of the RyR2, and they have shown extensive bilayer data consistent with FKBP12.6 depressing RyR2 openings that may represent diastolic SR Ca leak. Several major groups have reported that FKBP12.6 has no effect on RyR2 channel gating. In our above-mentioned study we found that increased saturation of RyR2 with extra FKBP12.6 could slightly, but significantly, reduce Ca sparks and increase SR Ca load. This effect is very small, much smaller than that in analogous experiments with calmodulin. That may be why some groups failed to detect FKBP12.6 effects, but is also consistent with FKBP12.6 overexpression studies (that amplify the endogenous FKBP12.6 effects) in which SR Ca sparks are decreased and SR Ca content is enhanced. I conclude that FKBP12.6 can indeed slightly depress RyR2 activity, but given the low fractional occupancy of RyR2 in control or HF animals, and lack of baseline alteration in FKBP12.6 knock-out mice (which via Marks’ model should exhibit full HF phenotype at baseline), I infer that FKBP12.6 affords only minor regulation.
Is RyR2-Mediated SR Ca Leak Increased in HF and by What Mechanism?

The first direct measurements of SR Ca leak in HF myocytes showed that diastolic SR Ca leak was increased at any given SR Ca content and that this enhanced leak could be suppressed by inhibition of CaMKII, but not by PKA inhibition. That is consistent with our controlled studies of PKA and CaMKII effects on RyR2 in phospholamban knockout mouse myocytes in which SERCA effects are abrogated (ie, CaMKII but not PKA induces robust increases in Ca sparks). Other groups have also reported increased SR Ca leak in various HF models, so I think that enhanced SR Ca leak is a bona fide finding in HF. I also think that CaMKII is important in that HF process, and CaMKII expression, activation state, and association with RyR2 are all increased in HF and that CaMKII inhibition can inhibit SR Ca leak. Reactive oxygen species can also directly activate RyR, and that may be part of the mechanism of enhanced SR Ca leak in HF. Moreover, CaMKII activation state can be elevated chronically by both autophosphorylation and reactive oxygen species, both of which may reinforce this SR Ca leak phenotype in HF.

Does Enhanced SR Ca Leak in HF Contribute to Dysfunction?

If the RyR2 has increased Ca sensitivity in HF (as I believe it does), how might that influence contractility? Eiser and Trafford have demonstrated that changes in RyR2 Ca sensitivity (eg, by caffeine) have only transient effects on systolic function due to a sort of autoregulation. That is, the first beat with higher Ca sensitivity does have greater SR Ca release, but that drives greater Ca extrusion from the cell via Na/Ca exchange, such that SR Ca uptake and content progressively decrease over a few beats. A new steady state is achieved at a lower SR Ca content and higher fractional SR Ca release, but where the Ca transient is unaltered (ie, driving the same Ca extrusion via Na/Ca exchange and where Ca influx and efflux are rebalanced). This lack of steady state effect of RyR2 modulation may function over a range of SR Ca loads, but if leak is too severe and SR Ca content too low, the Ca transient and contraction can be depressed. Enhanced diastolic SR Ca leak can push this in the extreme direction, but reduced SR Ca-ATPase function and increased Na/Ca exchange function also conspire to reduce SR Ca load in HF. In my mind, the effects on SERCA and Na/Ca exchange probably dominate with respect to the reduced systolic function in HF.

Triggered arrhythmias (eg, delayed after depolarizations), however, can be initiated by diastolic SR Ca release and propagating Ca waves, which cause inward Na/Ca exchange current and depolarization. There is compelling evidence that RyR2 point mutations that sensitize the channel to Ca-dependent activation are associated with the human arrhythmia catecholaminergic polymorphic ventricular tachycardia (CPVT) but not systolic dysfunction or HF. Thus, I also think that the increased diastolic SR Ca leak associated with HF is very likely to be arrhythmogenic, but may not contribute to systolic dysfunction (in a manner analogous to CPVT mutants).

Is Inhibition of SR Ca Leak a Valid Therapeutic Strategy?

Inhibiting diastolic SR Ca leak may be beneficial and therapeutic in HF, even if that involves neither FKBP12.6 binding to RyR2 nor RyR2 S2808 phosphorylation state. Acutely, this could decrease diastolic SR Ca leak and arrhythmogenicity. It could also diminish the intrinsic local positive feedback that Ca leak has on CaMKII activation that would perpetuate the leak. Blocking leak could also diminish Calmodulin dependent signaling to the nucleus via calcineurin and CaMKII, which are known to participate in transcriptional changes that drive or reinforce the hypertrophic and HF phenotype. Coming back to the papers at hand, it is hard to know which result is “correct” with respect to the impact of RyR2 S2808 phosphorylation (or not) on cardiac function and HF. However, on the basis of the foregoing discussion, I remain skeptical that S2808 plays an important role in either diastolic SR Ca leak or FKBP12.6 binding. Overall, I find the work of the Houser--Valdivia group, showing that S2808A had no effect on the inotropic effects of β-AR agonists or post-MI progression of HF, more consistent with my integrated conclusions from the above-focused questions. The impressive body of work from the Marks’ laboratory on this point paints a powerful and internally consistent picture, but the inability of many experienced labs around the world to reproduce key aspects of their model remains an issue.

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Disclosures

None.

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