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RYANODINE RECEPTOR PHOSPHORYLATION AND HEART FAILURE Phasing out S2808 and "criminalizing" S2814

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By the time the heart reaches the pathological state clinically recognized as *heart failure* (HF), it has undergone profound and often irreversible alterations in structure and function at the molecular, cellular and organ level. Although the etiologies of HF are diverse (hypertension, myocardial infarction, atherosclerosis, valvular insufficiency, mutations in genes encoding sarcomeric proteins, etc), some alterations are commonly found in most forms of HF, and they may account for the maladaptive structural remodeling and systolic dysfunction that characterize this syndrome. At the cellular level, there are well documented changes in ionic channel density and function (electrical remodeling), increased ROS production, mitochondrial dysfunction, imbalanced energy intake and consumption, genetic reprogramming, altered excitation-contraction coupling, and in general, dysregulation of a multitude of other processes and pathways that are essential for proper cardiac function.[1] Combined, this myriad of alterations leads to loss in contractility and ejection fraction, ventricular wall remodeling, increased vascular resistance, and dysregulated fluid homeostasis. In this issue of *Circulation Research*, Respress et al.[2] report that preventing phosphorylation of cardiac ryanodine receptors (RyR2) at a single residue, S2814, is sufficient to avert many of these alterations and improve cardiac function in HF. The results presented here follow a string of papers that touch on the delicate and controversial subject of ryanodine receptor phosphorylation and HF. They offer a new twist to a contentious story and attempt to reconcile many apparently contradicting results, but key issues remain.

Calcium "leak" in HF

Although, as mentioned above, the etiologies of HF are diverse, it appears that suppressing the dysfunction of a select group of biological and molecular signaling pathways may substantially improve or even reverse the cardiac deterioration observed in HF. For example, correcting the characteristically depressed sarcoplasmic reticulum (SR) calcium content of failing cardiomyocytes is a target of HF gene therapy.[3] SR calcium "leak", an operational term that indicates increased and untimely calcium release by RyR2s, also appears common to several models of HF[4,5] and it is reasonable to postulate that stemming off calcium "leak" may prevent the progression of cardiac malfunction in HF patients. However, a rationalized therapy towards this aim must be founded on the precise knowledge of the mechanisms leading to calcium leak, and work towards this knowledge has accelerated in recent years. Marks group, in a landmark publication in 2000 (ref. 6) and later in multiple other high-

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impact factor papers (many of them co-authored by Wehrens[7-10]) postulated that RyR2 "hyperphosphorylation" at S2808 by PKA was the primary mechanism leading to increased calcium "leak" in HF. This idea was initially appealing and fueled intensive research in the subject, but many groups failed to reproduce central tenets of this hypothesis.[reviewed in 11 and 12] The controversies surrounding the Marks-Wehrens hypothesis of increased calcium leak by hyperphosphorylation of RyR2-S2808 have been recently and comprehensibly reviewed by Bers.[13] Here I will focus on the modifications to this hypothesis as derived from the new findings of Respress et al.[2] Emerging points from these new findings will be the demotion of S2808, to intervene not as universal player in HF but only in selective forms of this syndrome, and the role of S2814 as pre-eminent generator of calcium leak that leads to arrhythmias and exacerbates other forms of HF. The "criminalization" of S2814 has begun in earnest.

CaMKII effect on calcium leak and the role of S2808 and S2814

Many studies have provided evidence that persistent CaMKII activity can lead to cardiac arrhythmias and promote HF.[14-16] Animals and patients with congestive HF display increased levels of CaMKII,[17,18] and overexpression of AC3-I, a peptide inhibitor of CaMKII, delays the onset of HF in mice.[19] There is also good agreement[4,20] (although not universal[21]) that CaMKII, and not PKA, increases calcium leak, and therefore, it is likely that the arrhythmogenic and deleterious activity of CaMKII in HF may be associated with this effect. Obviously, if PKA does not cause calcium leak directly, this by itself imposes insurmountable constraints on the Marks- Wehrens hypothesis that posits that PKA phosphorylation of RyR2-S2808 is responsible for the high calcium leak of HF. With the focus now on CaMKII, the obligated question is then, by what mechanisms CaMKII increases calcium leak from the SR? To increase calcium leak, the cell must either increase SR calcium content, and/or increase the activity of the RyR2 (albeit the latter alone would have only transient effects due to autoregulatory mechanisms[22]). Since both PKA and CaMKII increase SR calcium load by phosphorylating phospholamban (but at different residues) and relieving the inhibition it exerts on SERCA2a, the differential effect of these kinases must result from the regulation they exert on RyR2s. Wehrens group offers here[2] at least a partial explanation of this complex mechanism and, along with previous papers co-authored with Marks, these groups set specific roles for S2808 and S2814 on regulation of RyR2 activity and their protective effect (or lack thereof) in HF. In their view, PKA exclusively phosphorylates S2808 and dissociates FKBP12.6, which destabilizes the closed state of the channel and increases RyR2 activity, whereas CaMKII (almost) exclusively phosphorylates S2814, has no effect on FKBP12.6 binding, and equally activates RyR2s. In this issue, Respress et al.[2] report that preventing phosphorylation of S2814 (by genetic substitution of Ser by Ala, S2814A) protects against non-ischemic (pressure overload) HF but has no effect on ischemic HF; conversely, and against other data by the same groups, S2808 phosphorylation was not significantly different in non-ischemic HF, implying that it is relevant only in ischemic HF. This clean targeting of RyR2 phospho-epitopes by PKA and CaMKII and their nice "division of labor" for pathogenicity in distinct forms of HF would really simplify phosphorylation schemes and reconcile apparent contradictions. However, as is generally the case, the proposal appears oversimplified and almost too good to be true. Let's discuss each of the premises on which the Respress et al.[2] results have been interpreted and the problems associated with these premises.

One kinase = one site = one effect. Is it really that simple?

The RyR2 is a huge protein. It is assembled as a tetrameric complex of ~2 million Da, with each subunit composed of ~5,000 amino acids. Using canonical phosphorylation consensus and high confidence values, the RyR2 may be phosphorylated *in silico* at more than 100

sites by the combined action of PKA, CaMKII, PKG, and PKC, to name a few.[11] Granted, a "potential" phosphorylation site is very different than a demonstrated, physiologically-relevant phosphorylation site and it is possible that many of the predicted residues are not phosphorylated *in vivo.* Even then, several groups have demonstrated that CaMKII phosphorylates RyR2 with stoichiometry of at least 3 or 4 to 1 with respect to PKA.[23-26] This fact is by itself compelling evidence that there are *multiple* phosphorylation sites in RyR2. Now, let's make the optimistic assumption that *all* the PKA sites have already been mapped, and that S2808 and S2030 (ref. 27) are the only PKA sites. Taking into account the CaMKII:PKA phosphorylation ratio (3:1 or 4:1), this would then yield a minimum of ~6 – 8 CaMKII phosphorylation sites (per channel subunit!). In this perspective, it is almost disingenuous to label S2808 as "the" PKA site, and we may purposely deceive ourselves when we label S2814 "the" CaMKII site. Against this sense of pessimism and intractability, let's not forget that S2808 was actually discovered as a CaMKII site.[24] It is possible then that the number of CaMKII sites is smaller if only S2030 remains as a *bona fide* PKA site. Still, neither scheme supports one CaMKII site per channel subunit.

But let's go along for a moment with the possibility, however unlikely, that PKA phosphorylates S2808 only, and CaMKII phosphorylates S2814 only. When calling these sites by their distinctive numbers, it is easy to forget that these phospho-sites are only 6 residues apart, that is, a minuscule proportion (~0.000003%) in the context of the whole channel protein. How can the same reaction (phosphorylation) that occurs at sites so close to one another be differentially transmitted to the very distant gating domains of the channel? If these residues were lining the pore of the channel, where critical differences emerge by substituting one residue but not the neighboring one, then it would be easier to understand how S2808 and S2814 could transmit distinct signals. But both are part of a "phosphorylation hot spot", a cytoplasmic loop that contains additional potential phospho-sites[11] and that has been mapped to the external surface of the channel.[28] Marks and Wehrens groups have shown that phosphorylation of S2808A by CaMKII or of S2814A by PKA fully activate the channel.[7,9] At face value, this means that knocking out one phospho-residue does not cripple this "hot spot" and that phosphorylation of at least one residue in this external loop enables it to transmit conformational changes to the gating domains of the channel. Seen in this structural context in which the "hot spot" works in unison upon phosphorylation of at least one residue, it is very difficult (but not impossible) to accommodate the notion that phosphorylation of S2808 or S2814 alone dictates the differential response of the RyR2 to PKA and CaMKII.

An alternative model to explain differential PKA and CaMKII effects

An alternative model to explain the differential effect of PKA and CaMKII to elicit calcium leak from RyR2 that takes into account other phospho-sites is needed. Before formulating it, let's consider some important points. First, it is not difficult to assume that the role of the "phosphorylation hot spot" is to readily pick up signals from different kinases. The multi-valence of this "hot spot" is demonstrated so far by the fact that S2808 may be phosphorylated by CaMKII[24,25,26] and by PKA,[6,25,26] and its eagerness to undergo phosphorylation by the fact that S2808 is at least ~50% phosphorylated even at basal state[25-27,29,30] and phospho-signals from these sites may be readily detected upon β-adrenergic stimulation of the heart.[30,31] Second, if we accept the Shannon and Bers results that CaMKII, and not PKA, elicits calcium leak from the SR,[4,20] this obligatorily means that PKA phosphorylation of S2808 is not responsible for eliciting calcium leak (in direct conflict with the Marks-Wehrens hypothesis). In support of this notion, studies by the Houser and Valdivia groups have provided evidence that preventing S2808 phosphorylation has negligible impact on the β-adrenergic response of the heart and on the progression of non-ischemic and ischemic HF.[30-32] Third, another PKA site, S2030, largely ignored in the

Marks-Wehrens scheme, has been mapped and shown to activate channel openings[27] and although its place in the larger context of RyR2 phosphorylation has not been determined yet, I think it is illogical to assume that its existence is futile and that it contributes nothing to regulation of the channel. Thus, according to the preceding discussion, it is almost unsustainable to postulate that the differential effects of CaMKII and PKA to elicit calcium leak stems from their effects on the RyR2 "phosphorylation hot spot" alone. Instead, I would like to posit an alternative model that integrates findings by many of the above-referenced groups (Fig. 1). In this model, the surface domain of the RyR2 comprising residues 2804-2814 (mouse nomenclature) is an eager target for phosphorylation by PKA, CaMKII and probably other kinases (4 Ser/Thr).[11,24-26,29] Phosphorylation of this "hot spot" by either PKA or CaMKII (or both) "primes" the RyR2 for subsequent signals and is probably responsible for the coordinated openings in response to fast calcium stimuli detected in single channel recordings[33] and in cellular settings[34] (but this has yet to be demonstrated). The differential effect of PKA and CaMKII on RyR2 activity would then depend on the integrated response of the phosphorylated "hot spot" *and of additional* phosphorylation sites. For example, phosphorylation of S2808 and S2030 by PKA could coordinate channel openings in response to fast calcium stimuli, and phosphorylation of S2814 and other CaMKII site(s) could open RyR2s at diastolic [Ca^{2+}], which would translate in calcium leak. Examples of proteins acting as molecular switchboards in response to various degrees of phosphorylation are not unprecedented.[35] In fact, RyR2s are activated by phosphorylation and dephosphorylation as well[36,37] and their relative degree of phosphorylation determines a final functional output.[38] It is therefore conceivable that the complex response of RyR2s to any type of phosphorylation and the variable results obtained by investigators apparently using the same experimental conditions may be due to the variable degree of phosphorylation in which the RyR2s were found. Of course, until the 3D structure of the RyR2 is solved and we understand the mechanism by which the "phosphorylation hot spot" and other phospho-sites "talk" to the channel's gating domains this structurally-based model will remain speculative, but it at least takes into consideration compelling evidence on the existence of various phosphorylation sites and departs substantially from the simplified notion of one kinase = one site = one effect.

Now, let's go back to the results of Respress et al.[2] and consider them in this light. They found that preventing phosphorylation of S2814 *alone* mitigates non-ischemic HF induced by transverse aortic constriction (TAC) in mice. This implies that other CaMKII sites are not necessary to mitigate the CaMKII-induced calcium leak that they propose is responsible for the deleterious effect in WT mice subjected to TAC. If phosphorylation of the "hot spot" is compulsory to prime the RyR2 to process and discriminate other phosphorylation signals, then other residues in that "hot spot" must have been phosphorylated to fulfill this need. Surprisingly, S2808 was not significantly phosphorylated in this setting. This leaves a very difficult conundrum: if S2808 was not phosphorylated significantly and the other CaMKII sites are not necessary to stop calcium leak, how then can we explain the results of Respress et al.[2]? Of course there are always alternatives, and we would be inconsistent if we rigidly adhere to one model and fell into the dogmatism we are criticizing. The conclusions of Respress et al.[2] are in line with their findings, but at this point the numbers do not add up and it's obvious that the great complexity of this process (RyR2 phosphorylation) precludes simplified and neatly organized schemes. As a clear example of this, in the landmark paper by Marks group,[6] S2808 was found substantially hyperphosphorylated in tachypacing-induced failing dogs, also a non-ischemic model of HF. This does not fit well in the current scheme of Wehrens where S2808A protects against ischemic HF, but has no prominent role in non-ischemic HF.

In summary, CaMKII and PKA may have specific roles in calcium leak and, since they both increase SR calcium load, their differential effect likely resides on their effect on RyR2s.

However, the effect of PKA- or CaMKII-phosphorylation of RyR2s does not appear solved yet. Starting in 2000 and up to the present day, Marks and Wehrens have provided high-quality data in prominent journals aggressively pursuing the notion that PKA phosphorylates S2808 only, that CaMKII phosphorylates S2814 only, and that these sites alone integrate multiple signals to open RyR2s. Many key aspects of their general hypothesis including dissociation of FKBP12.6 by PKA phosphorylation of S2808, subconductance states as hallmarks of phosphorylation, and the prominent role of S2808 as promoter of arrhythmias and HF have not been confirmed by several groups. The present paper by the Wehrens group modifies slightly the original claim that S2808 was involved in ischemic and non-ischemic forms of HF and continues to shift the lion's share of pathogenicity to S2814. However, as discussed above, the Marks-Wehrens model largely ignores compelling data on the presence of multiple phosphorylation sites and the complexity they add to the finely graded response of RyR2s to phosphorylation.

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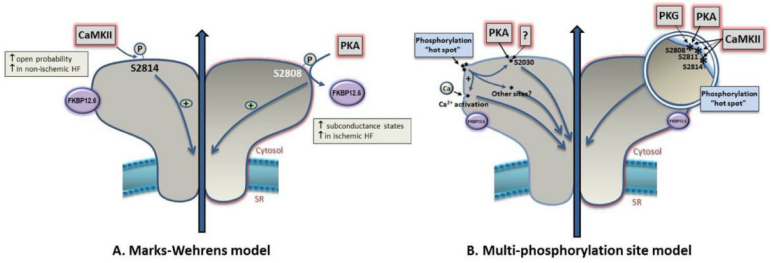

Fig. 1. Models of RyR2 modulation by phosphorylation

In the Marks-Wehrens model, S2808 is the only site phosphorylated by PKA, and S2814 by CaMKII. PKA phosphorylation of S2808 dissociates FKBP12.6, which destabilizes the closed state of the channel and induces subconductance states, eliciting calcium leak. Calcium leak from the SR then causes deleterious effects such as arrhythmias and worsening of (ischemic) HF. CaMKII phosphorylation of S2814 does not dissociate FKBP12.6 but also causes calcium leak. This leak is also arrhythmogenic, but is not relevant in ischemic HF, only in non-ischemic HF. In the multi-phosphorylation site model, S2808 and S2814 are part of a “phosphorylation hot spot” that is located in a protruding part of the channel, is targeted by several kinases, and may contain other phospho-epitopes not yet characterized. Phosphorylation of individual residues within this “hot spot” may be undistinguishable by the channel’s gating domains; instead, the differential regulation of PKA and CaMKII on channel gating may come about by the combined effect of each kinase on phospho-residues of the “hot spot” and other phosphorylation sites.