COMMENTARY

Cardiac ryanodine receptor phosphorylation: target sites and functional consequences

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A study by Xiao and co-workers in this issue of the *Biochemical Journal* demonstrates PKA (protein kinase A)-dependent phosphorylation of Ser-2030 on the cardiac ryanodine receptor (RyR2) that is activated by β -adrenergic agonists. They show that RyR2 phosphorylation at this site is not appreciably altered in heart failure samples, but retains PKA-dependence of phosphorylation. They contrast this with RyR2 phosphorylation at Ser-2808, a site previously reported to be the key and only PKA target site on RyR2. Here Ser-2808 phosphorylation was found to be relatively insensitive to either PKA activation or inhibition. These results add important new information to a highly controversial field. This issue is important because it is increasingly clear that altered regulation of the gating of the RyR2 sarcoplasmic reticulum Ca^{2+} -

release channel (e.g. by phosphorylation) is critically important in mediating altered diastolic sarcoplasmic reticulum Ca^{2+} release. This may contribute to both reduced cardiac function and arrhythmogenesis in humans carrying mutations in the RyR2 gene and with acquired heart failure of varied aetiology. This study brings some new answers, but also raises additional new questions that will require further investigation.

Key words: calcium, heart failure (HF), protein kinase A (PKA), ryanodine receptor phosphorylation (RyR phosphorylation), sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (SERCA), sarcoplasmic reticulum.

INTRODUCTION

Xiao et al. [1] show in this issue of the *Biochemical Journal* that PKA (protein kinase A)-dependent phosphorylation of the cardiac ryanodine receptor (RyR2) can occur at Ser-2030, and that this is dynamically regulated by β -adrenergic agonists in isolated myocytes. During normal cardiac EC (excitation–contraction) coupling a relatively small amount of Ca^{2+} enters the cell via the Ca^{2+} current (I_{Ca}) and this triggers the release of a larger amount of Ca^{2+} from the SR (sarcoplasmic reticulum) via RyR2 in a process called Ca^{2+} -induced Ca^{2+} release [2]. For the heart to relax between beats (diastole), Ca^{2+} must be transported out of the cytosol (allowing myofilament de-activation) and this is normally accomplished by the parallel function of SERCA2 (sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase 2) and sarcolemmal NCX (Na^{+} – Ca^{2+} exchange). In the steady state, the amount of Ca^{2+} influx and efflux (across both the SR and sarcolemma) must be in balance, so that neither the cell nor the SR progressively gains or loses Ca^{2+} . During diastole, there is also a small SR Ca^{2+} leak via RyR2 which may be visualized as Ca^{2+} sparks [2]. Again, depending on the competition between NCX and SERCA2, this leak can decrease SR Ca^{2+} content and reduce systolic function. The diastolic SR Ca^{2+} leak can also activate arrhythmogenic inward current via NCX, thereby producing delayed afterdepolarizations. Several human arrhythmias have been linked to specific RyR2 mutations {e.g. CPVT (catecholaminergic polymorphic ventricular tachycardia) [3]}, and the mutations studied so far have mostly increased Ca^{2+} sensitivity of RyR2 activation (e.g. [4]). These mutations may cause triggered arrhythmias by enhanced diastolic SR Ca^{2+} leak. In HF (heart failure), there is also enhanced diastolic SR Ca^{2+} leak which has been linked mechanistically to both lower SR Ca^{2+} content and arrhythmogenesis [5,6]. Thus alterations in cardiac myocyte Ca^{2+} regulation (and RyR2 function) are

centrally involved in both systolic dysfunction and arrhythmogenesis associated with HF in both animal models and in humans.

RyR2 is also a macromolecular complex containing PKA, phosphatases (PP1 and PP2a), FKBP12.6 (FK-506-binding protein 12.6), calmodulin, CaMKII (Ca^{2+} /calmodulin-dependent protein kinase II), phosphodiesterase 4D, triadin and junctin [7–9], all of which may regulate RyR2 gating. This provides a rich tableau of regulatory possibilities.

An extensive and elegant series of studies by Marks and colleagues (e.g. [7,9–12]) have developed a comprehensive mechanistic and attractive working hypothesis concerning SR Ca^{2+} leak in HF involving RyR2 phosphorylation (as follows). Owing to chronic β -adrenergic activation in HF, RyR2 is ‘hyperphosphorylated’ by PKA, causing dissociation of FKBP12.6 from RyR2 and increased RyR2 open probability (SR Ca^{2+} leak), resulting in reduced SR Ca^{2+} content and arrhythmogenic diastolic SR Ca^{2+} release. This enhanced RyR2 phosphorylation is facilitated by reductions in phosphatase and phosphodiesterase 4D association with the RyR2 in HF. They also presented results suggesting clearly that PKA phosphorylates RyR2 only at Ser-2809 (or at Ser-2808 depending on the species). This group also found that CaMKII phosphorylates RyR2 only at Ser-2815 (or at Ser-2814 depending on the species), which also activates RyR2 gating, but, in this case, not via FKBP12.6 dissociation. As attractive as this working hypothesis is, the attention it has received and its extensive testing by this group, it remains controversial. This is because numerous laboratory groups have found results that do not support different aspects of this hypothesis [1,13–17]. These include findings that RyR2 is not hyperphosphorylated by PKA in HF, that PKA-dependent phosphorylation does not cause FKBP12.6 release, that PKA activation does not increase basal RyR2 activation, that there are additional PKA sites on RyR2 and that Ser-2809 can also be phosphorylated by

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CaMKII or PKG (protein kinase G). Thus the sites of phosphorylation on RyR2 and the functional consequences require further clarification.

PHOSPHORYLATION OF RyR2 AT SER-2030

Xiao et al. [17] first identified that RyR2 could be phosphorylated at Ser-2030 in addition to two other previously identified sites (Ser-2809 and Ser 2815). In the latest issue of the *Biochemical Journal*, Xiao et al. [1] provide further compelling evidence that Ser-2030 in RyR2 in mouse, rat, rabbit and human can be phosphorylated by PKA. They show that Ser-2030 phosphorylation is extremely low before PKA activation, but increases rapidly upon PKA activation (and Ser-2030 is a poor substrate for CaMKII or PKG). In contrast, the Ser-2808 site was already substantially phosphorylated at basal conditions, but was increased further by PKA activation and could also be phosphorylated by CaMKII [17] and PKG (*in vitro*). In addition, the high basal Ser-2808 phosphorylation was not suppressed by inhibitors of PKA, CaMKII or PKG, but was blocked by staurosporine in HEK-293 (human embryonic kidney) cells expressing RyR2. This suggests that some other kinase may be important in basal phosphorylation at Ser-2808. Notably, they did see some increase in Ser-2808 phosphorylation in HF, but basal phosphorylation at Ser-2030 was not elevated. It is also fair to say that Ser-2808 phosphorylation could still have functional consequences in HF, even if it is not purely PKA-dependent. Phosphorylation at both sites could still be increased by PKA activation in HF (although the extent of enhancement at Ser-2808 was minor).

There are some important issues that were not addressed here. First, only Western blots were used, and the percentage of RyR2 phosphorylation or stoichiometry of the PO_4/RyR subunit was not quantified. This limits comparisons of absolute levels of phosphorylation at the two sites (antibody sensitivity and selectivity may differ, and detection may be non-linear). However, they showed previously that a 1:1 stoichiometry was achievable for Ser-2030 with PKA [17]. Moreover, they found that separate serine-to-alanine mutations of Ser-2030 and Ser-2808 each inhibited PKA-dependent ^{32}P incorporation similarly, and that the double mutant abolished ^{32}P incorporation induced by PKA. Secondly, there were no data to indicate the functional impact of RyR2 phosphorylation at Ser-2030. They showed previously no effect of a phosphomimetic mutant S2030D on the $[\text{Ca}^{2+}]$ -dependence of $[\text{H}]\text{ryanodine}$ binding [17]. Thus, while it is compelling that Ser-2030 is phosphorylated in response to PKA activation, it remains to be determined whether under physiological conditions this approaches 1:1 PO_4/RyR stoichiometry or has any functional impact.

Another unanswered question is why Wehrens et al. [11] could not detect PKA-dependent phosphorylation as ^{32}P incorporation (at Ser-2030) when they replaced wild-type RyR2 in mice with mutant S2808A RyR2 (via knock-in technology). Nor did mutation of Ser-2030 to alanine alter the phosphorylation of RyR2 compared with the wild-type [11]. They used these data to conclude that Ser-2808 is the only PKA site in RyR2. This is at odds with the study of Xiao et al. [17], where heterologous expression of an analogous S2808A RyR2 mutant still incorporated stoichiometric 1:1 PO_4/RyR upon PKA activation. It is possible that, in the study by Wehrens et al. [11], the conditions were such that they limited the ability to either phosphorylate Ser-2030 or to detect ^{32}P incorporation. Wehrens et al. [11] also performed functional experiments on RyR2 gating with these mutants. They found that PKA activated RyR2 gating in wild-type and S2030A mutant, but not S2808A mutant, channels (and

S2808D mutant channels were constitutively active). So, even if they had somehow failed to detect phosphorylation, there was still no detectable channel activation. Nevertheless, the compelling data from Xiao et al. [1,17] makes it inappropriate to dismiss the possibility that PKA-dependent RyR2 phosphorylation can occur at Ser-2030. If these laboratory groups swapped reagents and conditions, perhaps these differences could be reconciled.

Are there more phosphorylation sites on RyR2 than Ser-2030, Ser-2808 and Ser-2814? It seems likely that the only appreciable PKA-dependent phosphorylation occurs at Ser-2030 and Ser-2808, since mutating both serine sites to alanine in RyR2 abolishes PKA-dependent phosphate incorporation [17]. However, Rodriguez et al. [16] showed that the stoichiometry of RyR2 phosphorylation by CaMKII was 4-fold higher than for PKA, and this is consistent with greater amounts of ^{32}P incorporation via CaMKII than PKA [18,19]. Thus CaMKII can surely phosphorylate RyR2 at Ser-2814, and probably also at Ser-2808, and may also have as yet unidentified sites.

FUNCTIONAL CONSEQUENCE OF RyR SENSITIZATION

I will not review reports regarding the functional effects of RyR2 phosphorylation (which are also somewhat controversial). Rather, I will address the functional consequences of enhanced RyR2 Ca^{2+} sensitivity which have been suggested for some phosphorylation effects (PKA and CaMKII) and also for some CPVT mutations.

Increased RyR2 Ca^{2+} sensitivity may increase diastolic SR Ca^{2+} leak which may be arrhythmogenic and also contribute to reduced SR Ca^{2+} content (which would limit that leak). RyR2 sensitization would also increase the fractional SR Ca^{2+} release during EC coupling, such that a higher fraction of SR Ca^{2+} content is released. In the steady state, this may result in relatively normal systolic Ca^{2+} transients (as seen with low caffeine concentration [20]). As SR Ca^{2+} content declines (owing to greater fractional release and Ca^{2+} extrusion), diastolic leak should also decline and could return to the normal level (where it is equal and opposite to diastolic SERCA activity). Then the enhanced SR Ca^{2+} leak that really triggers spontaneous SR Ca^{2+} release may result from SERCA activation (via phospholamban phosphorylation) and acute increase in SR Ca^{2+} content. In this case, a Ca^{2+} -sensitized RyR2 alone is not arrhythmogenic, but the stimulation of SERCA and enhanced SR Ca^{2+} content that occurs concomitant with acute β -adrenergic activation may enhance triggered arrhythmias. This may explain why RyR mutations (and possibly enhanced phosphorylation) that sensitize RyR2 to Ca^{2+} are arrhythmogenic only during catecholaminergic stimulation (as in CPVT). This is even the case in the FKBP12.6-knockout mouse [12] which is remarkably not arrhythmogenic unless challenged with catecholamines.

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