Regulation of Ryanodine Receptors in the Heart

Stephan Lehnart, Andrew R. Marks

In response to exercise or other stresses, catecholamines are released into the circulation and within the heart. Catecholaminergic stimulation of β1-adrenergic receptors (β1ARs) in the heart increases heart rate (chronotropy) and contractility (inotropy), resulting in increased cardiac output during acute stress. Concurrent stimulation of β2-ARs dilates blood vessels, which increase blood flow to exercising muscles, thereby matching increased cardiac output to metabolic demands of the organs.

The inotropic mechanisms investigated by Valdivia and colleagues1 in the current issue of Circulation Research are essential for the acute stress-dependent increase of cardiac output. Each of the millions of muscle cells in the heart (cardiomyocytes) contribute to myocardial force development. Cardiomyocyte contraction is controlled by intracellular Ca2+ release through a process called excitation-contraction coupling (ECC) that involves the following steps: (1) an action potential (AP) depolarizes the cell membrane; (2) voltage-dependent plasma membrane L-type calcium channels (Ca1,2) opening results in a whole-cell inward Ca2+ current (ICa); (3) ICa activates cardiac ryanodine receptor (RyR2)/Ca2+ release channels located on the junctional sarcoplasmic reticulum (JSR), a process referred to as Ca2+ -induced Ca2+ release (CICR); (4) Ca2+ binds to troponin C (TroC) leading to cross-bridge formation between myosin and actin and contraction of the sarcomere. Cardiomyocyte relaxation is signaled by a return of intracellular [Ca2+]i to resting levels attributable to the following major mechanisms: (1) Ca1,2 inactivation; (2) RyR2 inactivation; (3) Ca2+ reuptake into the SR by SERCA2a pumps; and (4) Na+/Ca2+ exchange extrusion of Ca2+ to the extracellular space. Under resting conditions, net SR Ca2+ release contributes approximately 66% of Ca2+ necessary for myofilament activation in large mammals including humans, and approximately 90% in rodents.2

Stimulation of β-ARs in the heart results in cAMP-mediated activation of protein kinase A (PKA), which phosphorylates multiple intracellular proteins including Ca1,2,3 RyR2,4 and the SERCA2a inhibitor phospholamban (PLB).5 PLB phosphorylation at Ser16 by PKA dissociates PLB from SERCA2a and increases SR calcium uptake.5 Ca1,2 α-subunit phosphorylation at Ser1928 increases ICa amplitude and shifts activation threshold to more negative voltages.6 Using intact hearts or cardiomyocytes, multiple studies have found that RyR2 is phosphorylated by PKA during β-AR stimulation,7-15 and that PKA phosphorylation increases the single-channel open probability.4,7,8,16-18 As RyR2 mediates CICR, these observations suggest that PKA-mediated RyR2 phosphorylation may directly contribute to increased cardiac function during β-adrenergic stimulation (ie, during stress). Despite much evidence, the fundamental question of whether RyR2 PKA phosphorylation by itself increases SR Ca2+ release independent from ICa and SR Ca2+ load has been the subject of ongoing debate.

Characterizing the RyR2-specific component of SR Ca2+ release in intact cells during β-adrenergic stimulation has been a major challenge. Both SR Ca2+ load and ICa have been recognized as important mediators of SR Ca2+ release during inotropic modulation. ICa and [Ca2+]i, can be directly measured with patch-clamp and fluorescence indicators, respectively. Therefore activation of RyR2 during CICR in intact cells has been assessed by measuring intracellular Ca2+ release as a function of voltage-dependent ICa.6-10 Thus, the “normalized” increase of [Ca2+]i/ICa referred to as CICR “gain”, can be used to dissect the catecholamine-dependent role of RyR2 in CICR.

Previously, isolation of PKA effects on RyR2 function has been addressed indirectly using pharmacological modulators and [Ca2+]i loading protocols in cardiomyocytes. For example, using micromolar caffeine application in paced cardiomyocytes, Eisner and colleagues observed that the amplitude of intracellular [Ca2+]i and [Ca2+]i exchange extrusion of Ca2+ to the extracellular space. Under resting conditions, net SR Ca2+ release contributes approximately 66% of Ca2+ necessary for myofilament activation in large mammals including humans, and approximately 90% in rodents.2

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flash-photolysis of caged \( \text{Ca}^{2+} \) and convincingly demonstrated that independent from \( I_{\text{C}} \) and SR \( \text{Ca}^{2+} \) load, an increase in CICR occurs during \( \beta \)-AR stimulation.\textsuperscript{25} Their results provide support for a role of RyR2 PKA phosphorylation as a mechanism of increasing ECC gain.

**Significance of RyR2 PKA Phosphorylation in the Development of SR \( \text{Ca}^{2+} \) Leak During Heart Disease**

Defective intracellular \( \text{Ca}^{2+} \) homeostasis has been reported in heart failure (HF). Depressed SR \( \text{Ca}^{2+} \) uptake, storage, or release have all been documented in HF.\textsuperscript{26–28} SR \( \text{Ca}^{2+} \) load may be decreased because of depressed SERCA2a function or increased \( \text{Ca}^{2+} \) extrusion by increased Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger function contributing to reduced SR \( \text{Ca}^{2+} \) load.\textsuperscript{29,30} Moreover, increased diastolic SR \( \text{Ca}^{2+} \) leak and decreased SR \( \text{Ca}^{2+} \) load have been directly documented in HF models.\textsuperscript{31,32} We\textsuperscript{4,9,33} and others\textsuperscript{34–36} have previously demonstrated that chronic sympathetic hyperactivity in HF leads to increased protein kinase A (PKA) phosphorylation of RyR2 at Ser2808 and in decreased binding of the channel-stabilizing subunit calstabin2 (FKBP12.6). We have proposed that the depletion of calstabin2 from the RyR2 channel complex and PKA hyperphosphorylation of the channel results in a diastolic SR \( \text{Ca}^{2+} \) leak and contributes to HF progression, in part by contributing to SR \( \text{Ca}^{2+} \) depletion that impairs contractility.\textsuperscript{4} In agreement with this “leak hypothesis”, treatment with \( \beta \)-adrenergic blockers, which improve cardiac function and mortality in HF, prevents chronically increased RyR2 PKA phosphorylation, indirectly restores calstabin2 binding to RyR2, and reduces RyR2 channel leak.\textsuperscript{33,36}

To further investigate the specific role of PKA phosphorylation of RyR2-Ser2808 in HF progression, we generated an RyR2-S2808A knock-in model resulting in mice with RyR2 channels that cannot be PKA phosphorylated.\textsuperscript{37,38} We induced HF in these mice by ligating the left-anterior descending artery (LAD) and showed that the RyR2-S2808A knock-in protects against HF progression, providing support for the “leak” hypothesis that chronic PKA hyperphosphorylation of RyR2 causes a diastolic SR \( \text{Ca}^{2+} \) leak that contributes to SR \( \text{Ca}^{2+} \) depletion and impaired contractility that underlies HF progression.\textsuperscript{37,38} Benkusky et al generated a similar knock-in mouse to test our hypothesis that PKA phosphorylation of RyR2-S2808 is an important determinant of HF progression.\textsuperscript{1} However, instead of testing their mice using the post-MI HF model that we used, Benkusky et al used a model of cardiac hypertrophy induced by aortic banding. Unfortunately, the mice studied by Benkusky et al did not have HF, as evidenced by the lack of LV dilatation and the relatively preserved LV function in all groups (supplemental Table 1, available online at http://circres.ahajournals.org). Indeed, 4 weeks of aortic banding resulted in intermediate cardiac hypertrophy as evidenced by histology, and echocardiography showed normal function (Figure 1). After 11 weeks of banding, even though there was no evidence of heart failure per se, fractional shortening (FS) in WT banded animals was decreased by 19.2% (52.1±3.2% versus 42.1±1.2%, respectively; \( P<0.05 \)). However, 11 weeks after banding there was no decrease in FS in the RyR2-S2808A mice (FS=51.0±2.6% before banding versus 51.9±5.1% in banded RyR2-S2808A mice at 11 weeks). Thus, the RyR2-S2808A mice were protected against the mild decrease in cardiac function observed in the WT mice (supplemental Table 1). Therefore, despite the title of their article, the Benkusky et al data do not support the authors’ conclusion that HF progression is unmodified in the RyR2-S2808A mice.

**Physiological Role RyR2 Modulation by Catecholamines**

Benkusky et al also used the RyR2-S2808A knock-in mouse to study the role of PKA phosphorylation of RyR2 in regulating cardiac contractile function after \( \beta \)-adrenergic stimulation. We have previously identified RyR2-Ser2808 as the functionally important PKA site in RyR2. We showed that Ala for Ser substitution at Ser2808 resulted in ablation of PKA modulation of RyR2 channel function.\textsuperscript{4,7,38} We further showed that hearts from our global RyR2-S2808A knock-in mice are not different from age- and litter-matched WT control hearts in terms of baseline cardiac structure and function, a prerequisite for comparing modulation of cardiac function by catecholamines between groups.\textsuperscript{38} Thus, the RyR2-S2808A knock-in mouse can provide a unique opportunity to directly test the role of PKA phosphorylation of RyR2 in the regulation of cardiac contractility by catecholamines. Benkusky et al measured inotropic effects during \( \beta \)-adrenergic stimulation in cardiomyocytes using combined cell shortening and Ca\textsuperscript{2+} transient measurements. Surprisingly, Benkusky et al concluded that there were no significant differences between RyR2-S2808A and WT cells in terms of their response to \( \beta \)-adrenergic stimulation. Although cell shortening and Ca\textsuperscript{2+} transient amplitude have been used as measures of contractile function, there are important limitations to consider: Benkusky et al obtained their measurements using unloaded, isolated cardiomyocytes activated by electrical field stimulation, conditions that are quite different from the intact heart in vivo; the intracellular Ca\textsuperscript{2+} transient amplitude is the product of multiple Ca\textsuperscript{2+} transport mechanisms and depending on the experimental conditions may not specifically reflect RyR2 function. A more direct measure of RyR2 function, and of its specific role in ECC gain, requires normalization for \( I_{\text{C}} \), SR \( \text{Ca}^{2+} \) load, and \( [\text{Ca}^{2+}] \). In addition, physiological conditions such as temperature and pacing rate may influence cellular \( \text{Ca}^{2+} \) entry, release, and reuptake. Interestingly, Benkusky et al describe that after PKA activation, the amplitude of the intracellular Ca\textsuperscript{2+} transient and cell shortening were lower in RyR2-S2808A cells at higher, more physiological stimulation rates suggesting that there might indeed be a role for PKA phosphorylation of RyR2 in determining cardiac contractility despite their conclusion to the contrary.

Benkusky et al used saponin-permeabilized RyR2-S2808A cells to characterize ECC and found no significant differences in cAMP-stimulated spontaneous SR \( \text{Ca}^{2+} \) release events. Although these results are in agreement with Bers and colleagues using a similar approach,\textsuperscript{24} it is important to confirm these results using intact cells with preserved subcellular membrane microdomain structures and intact signaling pathways.
Benkusky et al further attempted to directly test the effects of PKA phosphorylation on single RyR2-S2808A channels in lipid bilayers. They concluded that “RyR2 fails to modify its activity in response to PKA phosphorylation at diastolic Ca\(^{2+}\)”, an expected finding because WT RyR2 channel open probability cannot be significantly increased under conditions that simulate diastole (eg, nanomolar cytosolic [Ca\(^{2+}\)] and millimolar cytosolic [Mg\(^{2+}\)])

However, they reported that recombinant RyR2-S2030D (designed to mimic constitutively PKA phosphorylated RyR2 at the S2030 site) exhibited no increased activity at different cytosolic (cis) Ca\(^{2+}\) concentrations as measured by \(\text{[H]}\)ryanodine binding.

In addition to identifying RyR2-Ser2808 as the major PKA phosphorylation site on the channel, we previously demonstrated its functional importance using single-channel measurements of recombinant and native RyR2 channels in planar lipid bilayers.\(^4,7,30\) Chen and colleagues have proposed that RyR2-Ser2030 is an alternative PKA phosphorylation site.\(^39\) However, they reported that recombinant RyR2-S2030D (designed to mimic constitutively PKA phosphorylated RyR2 at the S2030 site) exhibited no increased activity in a heterologous expression system.\(^40\)

Characterization of the molecular and cellular mechanisms of catecholamine-dependent modulation of cardiac contractile function remains challenging. Although use of a PKA phosphorylation-deficient RyR2-S2808A model represents an important technique, dissection of the specific role of RyR2-Ser2808 in cardiac inotropic regulation requires appropriate experimental design and data analyses. State-of-the-art experimental systems predict that the amplitude of \(I_{\text{Ca}}\) and SR Ca\(^{2+}\) load modulate the amount of RyR2 calcium release.\(^25,41\)

Therefore, important aspects to consider in the future study of \(\beta\)-adrenergic modulation of cardiomyocyte ECC gain are: (1) functional Cav1.2-RyR2 coupling; and (2) the relationship between RyR2-dependent Ca\(^{2+}\) release and SR Ca\(^{2+}\) load. Moreover, concomitant PKA and CamII phosphorylation of Cav1.2 and RyR2 may contribute to increased net SR Ca\(^{2+}\) release.\(^3,7,42,43\) Because the current study did not attempt simultaneous measurement of either Ca\(^{2+}\)-dependent or SERCA2a-dependent SR Ca\(^{2+}\) load, there are significant limitations to the authors’ conclusions. In summary, intracellular regulation of RyR2-dependent Ca\(^{2+}\) release by catecholamines is a complex mechanism underlying acute cardiac stress adaptation, elucidation of which is likely to lead to significantly better understanding of cardiac physiology and HF.

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