UltraRapid Communication

Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase II
Phosphorylation Regulates the Cardiac Ryanodine Receptor

Xander H.T. Wehrens, Stephan E. Lehnart, Steven R. Reiken, Andrew R. Marks

Abstract—The cardiac ryanodine receptor (RyR2)/calcium release channel on the sarcoplasmic reticulum is required for muscle excitation-contraction coupling. Using site-directed mutagenesis, we identified the specific Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) phosphorylation site on recombinant RyR2, distinct from the site for protein kinase A (PKA) that mediates the “fight-or-flight” stress response. CaMKII phosphorylation increased RyR2 Ca\textsuperscript{2+} sensitivity and open probability. CaMKII was activated at increased heart rates, which may contribute to enhanced Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release. Moreover, rate-dependent CaMKII phosphorylation of RyR2 was defective in heart failure. CaMKII-mediated phosphorylation of RyR2 may contribute to the enhanced contractility observed at higher heart rates. The full text of this article is available online at http://circres.ahajournals.org. (Circ Res. 2004;94:e61-e70.)

Key Words: ryanodine receptor ■ calcium ■ Ca\textsuperscript{2+}/calmodulin-dependent kinase II ■ protein kinase A ■ heart failure

The cardiac ryanodine receptor (RyR2) on the sarcoplasmic reticulum (SR) releases the calcium (Ca\textsuperscript{2+}) required for muscle contraction. On depolarization of the plasma membrane, Ca\textsuperscript{2+} enters the cell through voltage-gated Ca\textsuperscript{2+} channels. The ensuing Ca\textsuperscript{2+} influx triggers Ca\textsuperscript{2+} release from the SR through RyR2: a process referred to as Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR).\textsuperscript{1} The amplitude of the Ca\textsuperscript{2+} transient generated by SR Ca\textsuperscript{2+} release determines the contractile force in cardiomyocytes.\textsuperscript{2} Ryanodine receptor Ca\textsuperscript{2+} release can be increased by protein kinase A (PKA) after activation of β-adrenergic receptors on the plasma membrane.\textsuperscript{3,4} PKA phosphorylation of Ser\textsuperscript{2809} on RyR2 dissociates the channel-stabilizing protein FKBP12.6 (also known as calstabin2) from RyR2, which relieves inhibition of the channel and thus increases the RyR2 open probability.\textsuperscript{4,5} This evolutionarily conserved mechanism, part of the “fight-or-flight” stress response, allows for rapid enhancement of cardiac contractility and cardiac output during exercise or sudden stress.\textsuperscript{1}

RyR2 is a macromolecular signaling complex consisting of four RyR2 monomers, each of which is associated with proteins that regulate the channel function including the following: the channel-stabilizing subunit FKBP12.6; protein kinase A, which is bound to RyR2 via its targeting protein mAKAP; and protein phosphatases PP1 and PP2a that are bound to RyR2 via their targeting proteins spinophilin and PR130, respectively.\textsuperscript{6} Highly conserved leucine/isoleucine zippers (LIZ) in RyR2 form binding sites for LIZs present in the targeting proteins for the kinase (eg, PKA) and phosphatases (eg, PP1 and PP2A) that regulate RyR2 function.\textsuperscript{6} The direct targeting of kinases and phosphatases to the RyR channel allows for rapid localized regulation of the channel activity. There is also evidence that Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) associates with and regulates the activity of RyR2 in the heart.\textsuperscript{7–10} CaMKII plays an important role in regulating excitation-contraction (EC) coupling in the normal heart.\textsuperscript{11} In addition, activation of CaMKII at increased heart rates has been reported, and is believed to mediate increased Ca\textsuperscript{2+} release leading to increased contractile force (the positive force-frequency relationship).\textsuperscript{12} Finally, the increase in CaMKII activity reported in human heart failure has been proposed as a compensatory mechanism for the decreased cardiac contractility observed in failing hearts.\textsuperscript{13,14}

In this study, we show that CaMKII phosphorylates RyR2 at Ser\textsuperscript{2815}, which is distinct from the PKA phosphorylation site at Ser\textsuperscript{2809}. CaMKII phosphorylation of RyR2 at Ser\textsuperscript{2815} resulted in more active RyR2 channels. Identification of the principal CaMKII phosphorylation site on RyR2 and its functional role (as an activator of the channel) provide important insight into the mechanisms regulating Ca\textsuperscript{2+} release channel function.

Materials and Methods

Glutathione S-Transferase (GST) Fusion Proteins

Human RyR2 cDNA templates were amplified by PCR and subcloned into pGEX-2T (Amersham Pharmacia Biotech) for expression as glutathione S-Transferase (GST) fusion proteins (corresponding to amino acids 2776 to 2866). Mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene).\textsuperscript{4}

Expression of Recombinant RyR2

Wild-type and mutant RyR2 channels were expressed in HEK293 cells as previously described.\textsuperscript{15} Briefly, HEK293 cells were main-
CaMKII and PKA Phosphorylation of RyR2
Recombinant RyR2 (50 μg) was resuspended in 90 μL of CaMKII reaction buffer (50 mM Tris-HCl, 10 mM MgCl2, 2 mM ATP, 1:100 dilution), 10% for CaMKII, 15% for calmodulin, and FKBP12.6). CaMKII (250 U) was preactivated as per manufacturer’s instructions (New England Bio-labs) either in the presence or absence of the calmodulin inhibitor W-7 (25 μmol/L) or the CaMKII inhibitor KN-93 (1 μmol/L). The reaction buffer was supplemented with 100 μmol/L ATP, 1,2 μmol/L calmodulin, and 2 μmol/L CaCl2. CaMKII phosphorylation of immunoprecipitated RyR2 was performed by addition of γ32P-ATP to a final specific activity of 200 μCi/μmol. PKA phosphorylation of RyR2 was performed as previously described.5

Immunoprecipitations
RyR2 channels were immunoprecipitated from 200 μg of cell lysate homogenates or 100 μg cardiac SR membranes with anti-RyR antibody (5029) in 0.5 mL of 50 mM Tris-HCl buffer (pH 7.4), 0.9% NaCl, 0.5 mM EDTA, pH 7.5. The dephosphorylated SR preparation (AP, 1:100 enzyme to protein, New England Biolabs) in kinase buffer for 30 minutes at 37°C. The dephosphorylated SR preparation was then centrifuged at 10 000 g for 45 minutes. Pellets were resuspended in a buffer containing 250 mM/L sucrose.

Stoichiometric Phosphorylation of RyR2
Cardiac SR microsomes were pretreated with alkaline phosphatase (AP, 1:100 enzyme to protein, New England Biolabs) in kinase buffer for 30 minutes at 37°C. The dephosphorylated SR preparation was washed three times in kinase buffer to remove residual alkaline phosphatase. The stoichiometry of RyR2 phosphorylation by PKA or CaMKII was determined by [32P]-ATP incorporation into immunoprecipitated RyR2 (to ensure that we only measured [32P]-ATP incorporation into RyR2 monomers). The [32P]/RyR2 ratio was calculated by dividing [32P]-phosphorylation by the amount of high-affinity [3H]-ryanodine binding (there is one high-affinity ryanodine binding site per RyR2 tetramer) after normalizing for the amount of RyR2 immunoprecipitated from the SR membrane preparation.

For [3H]-ryanodine binding, 100 μg of protein were incubated with different concentrations of [3H]-ryanodine in 0.5 mL of binding buffer (170 mM/L KC1, 20 μmol/L CaCl2, 10 mM/L MOPS, pH 7.0 for 4 hours at 30°C). For nonspecific binding, 2 mM/L nonradioactive ryanodine was added. After incubation, the reaction mixture was filtered through Whatman filters and washed with ice-cold buffer (170 mM/L KC1, 10 mM/L MOPS). Bmax was determined by Scatchard analysis.

Single-Channel Recordings
Single-channel recordings of recombinant wild-type (RyR2-WT) or mutant RyR2 were performed and analyzed under voltage-clamp conditions as described.5 The trans chamber (1.0 mL) representing the intracellular compartment was connected to the head stage input of a bilayer voltage-clamp amplifier. The cis chamber (1.0 mL) representing the cytoplasmic compartment was held at virtual ground. Symmetrical solutions used are as follows (in mM/L): trans HEPS 250 and Ca(OH)2, 53, pH 7.35; cis HEPS 250, Tris 125, EGTA 1.0, and CaCl2 0.5, pH 7.35. Free [Ca2+] was calculated by CHELATOR software.46 At the conclusion of each experiment, ryanodine (5 μmol/L) or ruthenium red (20 μmol/L) was applied to confirm RyR2 channel identity. Channel traces represent individual experiments from untreated or in vitro PKA or CaMKII phosphorylated preparations as indicated.

Rabbit Langendorff Experiments
Studies were approved by the Institutional Animal Care and Use Committee of Columbia University. New Zealand White rabbits (Harlan, Indianapolis, Ind), weighing 2.0 to 3.0 kg, were anesthetized with pentobarbital (60 mg/kg IV) after injection of heparin (100 IU/kg IV). The heart was immediately excised, rinsed twice in ice-cold, Krebs-Henseleit (KH) solution (in mM/L: NaCl 137, KCl 5.4, HEPS 20, MgSO4 1.2, NaH2PO4 1.2, glucose 15, CaCl2 2.0, and NaF 1.0, saturated with O2, pH 7.4), and perfused by a modified Langendorf system with filtered KH buffer at a constant flow rate of 30 mL/min at 37°C. A saline-filled latex balloon (Kent Scientific) connected to a 3F micromanometer catheter (Millar Instruments) was inserted into the left ventricle via the mitral annulus for pressure measurements. The balloon was inflated to an initial diastolic pressure of 40 to 50 mm Hg and was kept isovolumic during the experiment. In some experiments, the KH solution was supplemented with either (1) the CaMKII inhibitor KN-93 (0.4 μmol/L; Calbiochem), or (2) the nonsensitive β-adrenergic receptor (β-AR) agonist isoproterenol (ISO; 0.1 μmol/ L). At completion of each experiment, the ventricular myocardium was flash frozen, and stored at –80°C.

Heart Failure Model
Twenty Sprague-Dawley rats (300 to 400 g; Harlan, Indianapolis, Ind) underwent left coronary artery ligation via left thoracotomy as previously described17 an additional eight rats underwent sham operations without coronary artery ligation (control group). After 1 week, echocardiography was performed on all 28 rats. Three months after coronary ligation, echocardiography was repeated and hemodynamic data were obtained.
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Data Analysis and Statistics
Data are presented as mean±SEM. The unpaired Student’s t test was used for statistical comparison of mean values between two experimental groups, and the one-way ANOVA test was used to compare mean values between three or more experimental groups. A value of P<0.05 was considered statistically significant.

Results
CaMKII Phosphorylates Serine2815 on RyR2
It has been suggested that PKA and CaMKII both phosphorylate the same residue on RyR2 (Ser2815),3,7 (Figure 1A). We have previously shown that substituting an alanine for Ser2809 in RyR2 in a GST fusion protein containing amino acids 2776 to 2866 from the human RyR2 isoform or in the full-length RyR2 protein eliminates PKA phosphorylation of RyR2, indicating that Ser2809 is the unique PKA site on the channel.4 However, GST-fusion protein RyR2-S2809A could still be phosphorylated by CaMKII (Figure 1B), indicating that the PKA and CaMKII sites on RyR2 are distinct. We noted that there is an S-x-D motif adjacent to the PKA site on RyR2 that could serve as a substrate for CaMKII phosphorylation.4,9,10 The peptide used to identify the CaMKII site by Jones and colleagues7 contains, in addition to Ser2809, one potential CaMKII site, S2815-V-D (Figure 1A), and a second potential CaMKII site is located at Ser2835. We found that GST-fusion protein mutant RyR2-S2815A could not be phosphorylated by CaMKII, whereas the RyR2-S2835A mutation was able to be CaMKII phosphorylated (Figure 1B). In order to determine whether Ser2815 was the unique PKA site on recombinant RyR2, full-length human RyR2 cDNA was expressed in HEK293 cells. SR microsomes were prepared, and RyR2 were immunoprecipitated with an anti-RyR-5029 antibody. RyR2-S2815A channels could not be phosphorylated by CaMKII (Figure 1C), confirming that Ser2815 is the unique CaMKII site on recombinant RyR2.

Distinct Mechanisms for CaMKII and PKA Regulation of RyR2
We have previously demonstrated that PKA phosphorylation of RyR2 at Ser2809, which is part of the fight-or-flight response, dissociates the regulatory subunit FK506 binding protein (FKBP12.6) from the RyR2 macromolecular complex.4 RyR2-S2809D channels mimic constitutively phosphorylated RyR2 and were not able to bind FKBP12.6 when cotransfected in HEK293 cells (Figure 1C, bottom gel). In contrast, RyR2-S2815D channels, which mimic constitutively CaMKII phosphorylated RyR2, were able to bind FKBP12.6 (Figure 1C). Phosphorylation of wild-type RyR2 channels by exogenous PKA or CaMKII showed that PKA but not CaMKII phosphorylation of RyR2 dissociates FKBP12.6 from the channel (data not shown). Taken together, these data show that PKA and CaMKII phosphorylate RyR2 at adjacent but distinct sites, and whereas both can lead to activation of RyR2, only PKA phosphorylation causes dissociation of FKBP12.6 from the channel.

CaMKII activity coimmunoprecipitated with RyR2, and we found that the CaMKII-δ isoform was a component of the RyR2 macromolecular complex, consistent with earlier reports that CaMKII-δ copurifies with RyR221 (Figure 1D). To demonstrate that the CaMKII in the RyR2 complex was active, we added Ca2+ and CaM to immunoprecipitated RyR2, in the absence of exogenous CaMKII, and showed that RyR2 could be CaMKII phosphorylated (Figure 1E), indicating that the RyR2 macromolecular complex contains enzymatically active CaMKII.

CaMKII Phosphorylation of RyR2 Increases Open Probability
Studies examining the functional effects of CaMKII phosphorylation of RyR2 have yielded conflicting results.7,9,19,20 To resolve this controversy, we utilized the recombinant wild-type and mutant RyR2 that are either unable to be phosphorylated by CaMKII or mimic constitutively CaMKII phosphorylated RyR2. Recombinant RyR2 channels were phosphorylated by PKA or CaMKII, and subsequently incorporated into planar lipid bilayers. CaMKII phosphorylation of RyR2 increased channel activity comparable to the effect of PKA phosphorylation of RyR2 but without inducing partial channel openings (eg, subconductance levels) (Figure 2A). Similar results were obtained when RyR2 purified from canine heart was phosphorylated by PKA or CaMKII (data not shown). PKA phosphorylation of the mutant RyR2-S2809A that lacks the PKA phosphorylation site (Ser2809) did not increase open probability (Po) compared with RyR2-WT, whereas PKA phosphorylated RyR2-S2815A channels did show increased Po and subconductance levels (Figure 2B). RyR2-S2809D channels, which mimic constitutively PKA phosphorylated RyR2, displayed increased Po, similar to PKA phosphorylated RyR2-WT (or RyR2-S2815A) channels. In contrast, CaMKII phosphorylation of RyR2-S2815A channel did not increase Po whereas RyR2-S2809A channels could be activated by CaMKII (Figure 2B, bottom). RyR2-S2815D channels, mimicking constitutively CaMKII phosphorylated RyR2, displayed increased Po similar to CaMKII phosphorylated wild-type RyR2 (or RyR2-S2809A). Amplitude histograms show that in contrast to PKA phosphorylation (eg, RyR2-S2809D), CaMKII phosphorylation (RyR2-S2815D) does not induce subconductance openings (Figure 2C). Moreover, CaMKII phosphorylation of RyR2 increased sensitivity of the channel to Ca2+-dependent activation at [Ca2+] between 100 and 1000 nmol/L (Figure 2D).

CaMKII Phosphorylation Activates RyR2 During Faster Heart Rates
Because CaMKII is known to be able to decode the frequency and amplitude of Ca2+ signals, we sought to define the effects of CaMKII on RyR2 because RyR2 is a Ca2+ activated channel. The rate of increase in contractility associated with increased heart rates was inhibited by CaMKII inhibitors KN-93 (Figure 3A) and W-7 (data not shown). In addition, the cardiac contractility (measured as developed ventricular pressure) was enhanced at higher heart rates and this effect was also blocked by inhibition of CaMKII (Figure 3A).

We examined the single-channel properties of RyR2 incorporated into planar lipid bilayers that were isolated from nonpaced Langendorff-perfused rabbit hearts that beat spontaneously at 10 to 100 bpm and paced (HR 200 bpm) hearts. The...
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enhanced CaMKII activity in the RyR2 macromolecular complex.

It has been previously shown that in response to a sudden change in heart rate, phosphorylation of phospholamban (PLB) by CaMKII occurs at Thr17, in the absence of PLB-Ser16 phosphorylation by PKA, suggesting that activation of CaMKII is part of a distinct signaling pathway triggered by increased heart rate.22 Therefore, we assessed the phosphorylation status of PLB in response to an increased heart rate in the rabbit Langendorff model. In contrast to PLB-Ser16 phosphorylation, PLB-Thr17 phosphorylation was increased in response to an increased heart rate (Figures 3H and 3I), and this increased phosphorylation of PLB was blocked by the CaMKII inhibitors (KN-93 and W-7).

Stoichiometric CaMKII Phosphorylation of Native RyR2

Previous studies have reported that CaMKII may phosphorylate more than one CaMKII site on RyR2.9,23–26 Rabbit cardiac SR membranes were first dephosphorylated using alkaline phosphatase (Figure 4A). Complete dephosphorylation was confirmed using an antibody that specifically recognizes the dephosphorylated RyR2-Ser2809. Cardiac SR membranes were subsequently washed three times using kinase buffer to remove residual alkaline phosphatase. The dephosphorylated RyR2 were phosphorylated in the presence of 32P-ATP by (1) exogenously added PKA, (2) endogenous PKA targeted to RyR2 activated by camp, (3) exogenously added CaMKII, and (4) endogenous CaMKII targeted to RyR2 activated by Ca2+ and calmodulin (CaM) (Figure 4A).

In order to determine the stoichiometry of PKA and CaMKII phosphorylation of RyR2, the amount of RyR2 in 100 μg of SR was quantified using [3H]-ryanodine binding. We subsequently compared the amount of immunoprecipitated RyR2 to a known amount of RyR2 (in the SR membrane preparation) using Western blotting. Quantification of the amount of 32P-ATP incorporated into immunoprecipitated RyR2 indicated that PKA incorporates 1.00 ± 0.18 mol of phosphate per mol of RyR2 monomer, whereas CaMKII incorporates 1.50 ± 0.07 mol of phosphate per mol of RyR2 monomer (Figure 4B). Pretreatment with nonradioactive PKA did not change the amount of 32P-ATP incorporated into RyR2 by CaMKII (1.51 ± 0.01).

Defective CaMKII Regulation of RyR2 in Failing Hearts

Cardiac muscle from humans with failing hearts typically shows a decreased or inverse force-frequency relationship resulting in a decrease in contractile performance at higher heart rates for reasons that are not well understood.27 In this study, we showed that in control (sham-operated; n = 8) animals the level of CaMKII phosphorylation of RyR2-Ser2815 increased proportionately with elevated heart rate (r=0.905; Figure 5A). However, in rats with heart failure after myocardial infarction (n=20), the relation-
ship between elevated heart rate and increased CaMKII phosphorylation of RyR2 was absent \( r = 0.457 \); Figure 5B).

**Discussion**

This study identifies the principal CaMKII phosphorylation site on the cardiac ryanodine receptor (RyR2) using site-directed mutagenesis and shows that phosphorylation at this site activates the channel. We showed that Ser\(^{2815}\) is the principal CaMKII phosphorylation site on recombinant RyR2, whereas Ser\(^{2809}\) is the target of phosphorylation by PKA. Previous studies have suggested that Ser\(^{2809}\) may be the phosphorylation site of both PKA and CaMKII based on sequencing of tryptic phosphopeptides\(^{3,7}\) or a phosphoepitope-specific antibody.\(^{26}\) Our studies are the first to use site-directed mutagenesis of the
full-length RyR2 channel to show that there are two distinct phosphorylation sites for PKA and CaMKII, because the mutation RyR2-S2809A only prevents PKA phosphorylation, and the mutation RyR2-S2815A only abolishes CaMKII phosphorylation of RyR2, respectively. Moreover, we were also able to show that both serines can be independently phosphorylated by PKA and CaMKII, respectively, using phosphoepitope antibodies specific for either phosphorylated Ser2809 or Ser 2815 on RyR2 (Figure 3). Thus, the present study is the first to use site-directed mutagenesis in the full-length recombinant RyR2 to unambiguously identify the CaMKII site on RyR2. The presence of the CaMKII site on RyR2 adjacent to the PKA site (only six amino acid residues away) may explain why previous studies mistakenly reported that PKA and CaMKII phosphorylate the same site on RyR2.3,7

Phosphorylation of native RyR2 from rabbit heart using CaMKII incorporated a maximum of 1.5 moles of phosphate per mol of RyR2 monomer. Our stoichiometry data are consistent with most other published studies that have reported a CaMKII phosphorylation stoichiometry of 0.2 to 2.0 moles of phosphate per mole of RyR monomer.9,23–25 In contrast, Rodriguez et al26 recently reported that CaMKII may phosphorylate up to four sites on RyR2. These data should be interpreted with caution because this group did not assess the amount of RyR in their samples using [3 H]-ryanodine, but rather normalized the amount of 32 P-ATP incorporated into RyR2 by CaMKII relative to the amount incorporated by PKA, which may have led to an overestimate of the CaMKII sites. In addition, the short duration of the PKA phosphorylation (1 minute) may have induced incomplete phosphorylation, because Jiang et al28 have previously reported that under similar conditions stoichiometric PKA phosphorylation may take at least 3 minutes. Thus, our results suggest that besides Ser2815, a second CaMKII site may be partially phosphorylated in native RyR2 under pharmacological conditions, whereas in recombinant RyR2 only Ser2815 is subject to CaMKII phosphorylation. The exact nature of this putative second CaMKII site and the physiological significance of this site after changes in heart rate remain to be determined.

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The cytoplasmic domain of RyR2 serves as a scaffold for PKA and protein phosphatases PP1 and PP2A, which modulate the function of the channel via PKA phosphorylation of Ser2809. Previous studies have suggested that CaMKII may be part of the RyR2 macromolecular complex based on copurification25,29,30 and the presence of endogenous CaMKII activity associated with purified RyR2.25,30 In the present study,
we show that CaMKII is indeed part of the RyR2 macromolecular complex using communoprecipitations, in agreement with recent studies by Zhang et al.4,5

Our data show that PKA phosphorylation of recombinant RyR2 increases channel activity and leads to the dissociation of FKBP12.6 from the channel complex, and agree with previous similar findings obtained by us and by others using native RyR2 preparations.4,5,24,31,32 However, several laboratories have challenged the idea that PKA phosphorylation leads to increased channel activity33,34 and FKBP12.6 release.33,35 Li et al35 examined the effects of PKA phosphorylation of RyR2 in normal cardiomyocytes under diastolic conditions when the RyR2 has to remain tightly shut in order to avoid triggering arrhythmias. They showed no increase in Ca2+ sparks under these conditions as would be expected, but this same group more recently showed that PKA phosphorylation of RyR2 does increase EC coupling gain by enhancing the rate of RyR2 mediated SR Ca2+ release during the early phase of EC coupling,36 which is entirely consistent with our model.4

Terentyev et al37 reported that protein phosphatase 1 (PP1) may increase RyR2 activity. These results, however, contrast with several other studies that reported that PP1 decreases RyR activity38,43 and that PKA phosphorylation increases RyR activity.4

We have previously shown that PKA phosphorylation results in the dissociation of FKBP12.6 from the macromolecular complex.1,4 In contrast, other groups have shown that FKBP12.6 may bind to PKA phosphorylated RyR2 under certain experimental conditions.28,34 Recent data from our group provide an explanation for these apparent different findings. PKA phosphorylation of RyR2 (or the mutation S2809D in RyR2) decreases the binding affinity of FKBP12.6, resulting in the release of FKBP12.6 from the RyR2 complex. However, when FKBP12.6 is present in very high concentrations (eg, when it is coexpressed with RyR2 under nonstoichiometric conditions as in Stange et al44 and Xiao et al44, or added in excess amounts as in Xiao et al44), FKBP12.6 may still be able to bind to PKA phosphorylated RyR2 or RyR2-S2809D because it overcomes the shift in Kd induced by PKA phosphorylation of Ser2809.45 Because FKBP12.6 concentrations in mammalian hearts are relatively low (in the order of 200 to 400 nmol/L), PKA phosphorylation (for example during stress or exercise) will result in partial dissociation of FKBP12.6 from RyR2.5,45 Thus, in performing experiments to examine the affects of PKA phosphorylation of RyR2 or mutation (eg, RyR2-S2809D) on the binding of FKBP12.6 to the channel, we are careful to maintain physiological ratios of FKBP12.6 to RyR2. Failure to do so may explain different findings reported by other groups.34,44

Contradictory results have also been reported regarding the functional effects of CaMKII phosphorylation of RyR2 with some studies showing activation7–9 and some inactivation.19,20 Witcher et al17 used single-channel recordings to show that CaMKII phosphorylation of RyR2 produces long openings and an increased open probability, a finding we confirmed using CaMKII phosphorylated RyR2-WT channels and RyR2-S2815D mutant channels (Figure 2). Recently, Maier et al46 demonstrated that cardiomyocytes isolated from mice overexpressing cytoplasmic CaMKIIδ, display an increased Ca2+ spark frequency, which could be blocked by CaMKII-inhibitor KN-93. Together with our data, this suggests that CaMKII phosphorylation increases the open probability of RyR2 and leads to enhanced intracellular SR Ca2+ release.56,47

Contractility increases with increasing heart rate, known as the Bowditch phenomenon or positive force-frequency (staircase) relationship.48,49 The positive force-frequency relationship may require alterations of both intracellular Ca2+ release and reuptake into the SR, to provide sufficient time for diastolic filling of the ventricle at higher heart rates.50 An increase in the frequency of intracellular Ca2+ concentration elevations during increased heart rate activates CaMKII,51 which is thought to mediate the increased Ca2+ reuptake into the SR.52 It has been proposed that speeding of Ca2+ reuptake is mediated by Ca2+/calmodulin-dependent kinase II (CaMKII) phosphorylation of phospholamban (PLB) at Thr17, which would increase SERCA2a activity.22,53 However, frequency-dependent acceleration of relaxation and a positive force-frequency response are intact in PLB-deficient mice and remain sensitive to CaMKII inhibition by KN-93.30,54 suggesting additional mechanisms may contribute to enhanced intracellular Ca2+ reuptake at faster heart rates.

Although some studies have suggested that Ser16-phosphorylation of phospholamban (PLB) precedes or is required for Thr17-PLB phosphorylation,55,56 other studies have provided compelling evidence that PLB can be phosphorylated at Thr17.
by CaMKII, independently of prior phosphorylation at Ser2 by PKA.22,27 Our data using phosphoepitope-specific antibodies for PLB and RyR2 suggest that independent phosphorylation by PKA and CaMKII is indeed possible in the setting of β-AR stimulation or increases in heart rate, respectively.

Shortening of the Ca2+ transient at faster heart rates may also be caused by a decrease in time to peak [Ca2+], which may reflect an enhanced Ca2+ sensitivity of the SR Ca2+ release mechanism.58 Although biochemical studies have shown that CaMKII can phosphorylate RyR2, the site of CaMKII phosphorylation of the receptor and the functional role of CaMKII phosphorylation during elevated heart rates have not been reported.3 Our data suggest that at increased heart rates, the increase in time-averaged [Ca2+], leads to activation of CaMKII associated with RyR2.21 The increased Ca2+ sensitivity of CaMKII-phosphorylated RyR2 may lead to a faster (and larger) upstroke of the Ca2+ transient.12,46 Moreover, the relationship between elevated heart rate and increased CaMKII phosphorylation of RyR2 was not observed in failing rat hearts. Thus, altered CaMKII phosphorylation of RyR2-Ser2841 could contribute to the loss of the force-frequency relationship in heart failure.27

The present study suggests that two distinct phosphorylation-dependent pathways are available for activating RyR2: one is activated by increased heart rate and involves Ca2+/CaMKII-dependent phosphorylation of RyR2 at Ser2841; the other is activated by the sympathetic nervous system as part of the fight-or-flight stress response and involves β-adrenergic receptor/PKA phosphorylation of RyR2 at Ser2809 (Figure 6).4 CaMKII phosphorylation does not lead to dissociation of the channel stabilizing protein FKBP12.6 from the RyR2 channel complex. Therefore, increasing RyR2 channel activity through CaMKII phosphorylation, which likely accompanies activation of RyR2 by PKA phosphorylation, may prevent dissociation of excessive amounts of FKBP12.6 from the RyR2 macromolecular complex, thus preventing a reduction in coupled gating59 and an increased propensity for cardiac arrhythmias.5

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