

## Ca<sup>2+</sup>/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<sup>2+</sup>/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<sup>2+</sup> sensitivity and open probability. CaMKII was activated at increased heart rates, which may contribute to enhanced Ca<sup>2+</sup>-induced Ca<sup>2+</sup> 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<sup>2+</sup>/calmodulin-dependent kinase II ■ protein kinase A ■ heart failure

The cardiac ryanodine receptor (RyR2) on the sarcoplasmic reticulum (SR) releases the calcium (Ca<sup>2+</sup>) required for muscle contraction. On depolarization of the plasma membrane, Ca<sup>2+</sup> enters the cell through voltage-gated Ca<sup>2+</sup> channels. The ensuing Ca<sup>2+</sup> influx triggers Ca<sup>2+</sup> release from the SR through RyR2s: a process referred to as Ca<sup>2+</sup>-induced Ca<sup>2+</sup> release (CICR).<sup>1</sup> The amplitude of the Ca<sup>2+</sup> transient generated by SR Ca<sup>2+</sup> release determines the contractile force in cardiomyocytes.<sup>2</sup> Ryanodine receptor Ca<sup>2+</sup> release can be increased by protein kinase A (PKA) after activation of  $\beta$ -adrenergic receptors on the plasma membrane.<sup>3,4</sup> PKA phosphorylation of Ser<sup>2809</sup> 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.<sup>4,5</sup> 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.<sup>1</sup>

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.<sup>6</sup> 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.<sup>6</sup> 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<sup>2+</sup>/calmodulin-dependent protein kinase II (CaMKII) associates with and regulates the activity of RyR2 in the heart.<sup>7–10</sup> CaMKII plays an important role in regulating excitation-contraction (EC) coupling in the normal heart.<sup>11</sup> In addition, activation of CaMKII at increased heart rates has been reported, and is believed to mediate increased Ca<sup>2+</sup> release leading to increased contractile force (the positive force-frequency relationship).<sup>12</sup> 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.<sup>13,14</sup>

In this study, we show that CaMKII phosphorylates RyR2 at Ser<sup>2815</sup>, which is distinct from the PKA phosphorylation site at Ser<sup>2809</sup>. CaMKII phosphorylation of RyR2 at Ser<sup>2815</sup> 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<sup>2+</sup> 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).<sup>4</sup>

#### Expression of Recombinant RyR2

Wild-type and mutant RyR2 channels were expressed in HEK293 cells as previously described.<sup>15</sup> Briefly, HEK293 cells were main-

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tained in T175 flasks in minimum Eagle's medium containing 10% bovine serum and were passed every 3 to 4 days. One T175 flask (50% confluent) was transfected with 20  $\mu\text{g}$  of RyR2 cDNA and 2.5  $\mu\text{g}$  of FKBP12.6 cDNA using the  $\text{Ca}^{2+}$  phosphate precipitation method.<sup>15</sup> Forty-eight hours after transfection, cells were washed twice, scraped into phosphate-buffered saline, and pelleted by centrifugation at 2500g for 5 minutes. After resuspending the pellet in 0.5 mL of 20 mmol/L HEPES-NaOH, pH 7.5, containing protease inhibitors (complete EDTA-free inhibitors from Roche Molecular Biochemicals), cells were allowed to swell for 30 minutes on ice before lysis through a 25G needle. Cell homogenates were diluted with an equal volume of ice-cold medium containing 500 mmol/L sucrose and 10 mmol/L HEPES, pH 7.2, and centrifuged at 10 000g for 15 minutes. Supernatants were recovered and centrifuged at 100 000g for 45 minutes. Pellets were resuspended in a buffer containing 250 mmol/L sucrose.

### CaMKII and PKA Phosphorylation of RyR2

Recombinant RyR2 (50  $\mu\text{g}$ ) was resuspended in 90  $\mu\text{L}$  of CaMKII reaction buffer (50 mmol/L Tris-HCl, 10 mmol/L  $\text{MgCl}_2$ , 2 mmol/L DDT, 0.1 mmol/L  $\text{Na}_2\text{EDTA}$ , pH 7.5). CaMKII (250 U) was preactivated as per manufacturer's instructions (New England Biolabs) either in the presence or absence of the calmodulin inhibitor W-7 (25  $\mu\text{mol/L}$ ) or the CaMKII inhibitor KN-93 (1  $\mu\text{mol/L}$ ). The reaction buffer was supplemented with 100  $\mu\text{mol/L}$  ATP, 1.2  $\mu\text{mol/L}$  calmodulin, and 2 mmol/L  $\text{CaCl}_2$ . CaMKII phosphorylation of immunoprecipitated RyR2 was performed by addition of  $\gamma\text{P}^{32}$ -ATP to a final specific activity of 200  $\mu\text{Ci}/\mu\text{mol}$ . PKA phosphorylation of RyR2 was performed as previously described.<sup>5</sup>

### Immunoprecipitations

RyR2 channels were immunoprecipitated from 200  $\mu\text{g}$  of cell lysate homogenates or 100  $\mu\text{g}$  cardiac SR membranes with anti-RyR antibody (5029) in 0.5 mL of 50 mmol/L Tris-HCl buffer (pH 7.4), 0.9% NaCl, 0.5 mmol/L NaF, 1.0 mmol/L  $\text{Na}_3\text{VO}_4$ , 0.5% Triton-X100, and protease inhibitors overnight at 4°C. The samples were incubated with protein A Sepharose beads (Amersham Biosciences) at 4°C for 1 hour after which the beads were washed three times with kinase buffer (8 mmol/L  $\text{MgCl}_2$ , 10 mmol/L EGTA, and 50 mmol/L Tris/piperazine-*N,N'*-bis-2-ethanesulfonic acid, pH 6.8).

### Immunoblots

Microsomes (50  $\mu\text{g}$ ) were size fractionated by SDS-PAGE (6% for RyR, 10% for CaMKII, 15% for calmodulin, and FKBP12.6). Proteins were transferred to nitrocellulose overnight at 50 V. Immunoblots were blocked with 5% milk in TBS-Tween and incubated with primary antibody (anti-FKBP12.6, 1:1000), anti-RyR (5029, 1:3000), anti-RyR2-pSer<sup>2809</sup> (1:1000),<sup>5</sup> anti-dephospho-RyR2-Ser<sup>2809</sup> (Badrilla, 1:4000), anti-phospholamban (PLB) (Affinity BioReagents, 1:5000), anti-PLB-pSer<sup>16</sup> (Research Diagnostics, 1:5000), anti-PLB-pThr<sup>17</sup> (Badrilla, 1:5000), anti-calmodulin (N-19, Santa Cruz Biotechnology, 1:500), anti-CaMKII- $\delta$  (A-17, Santa Cruz Biotechnology, 1:250), and anti-CaMKII-pThr<sup>287</sup> (Affinity BioReagents, 1:1000), for 1 hour at room temperature. The RyR2-pSer<sup>2815</sup> phosphoepitope-specific anti-RyR2 antibody is an affinity-purified polyclonal rabbit antibody generated using the peptide CSQTSQV-(pS)-VD corresponding to RyR2 CaMKII phosphorylated at Ser<sup>2815</sup>. After incubation with HRP labeled anti-rabbit IgG (1:5000 dilution, Transduction Laboratories), immunoblots were developed using ECL (Amersham Pharmacia).

### CaMKII Activity

RyR2 were immunoprecipitated and CaMKII activity associated with the RyR2 macromolecular complex was determined using a commercial assay (Upstate Biotechnology). Endogenous phosphorylation of proteins in the RyR2 complex was subtracted by examining immunoprecipitates in the absence of CaMKII substrate.

### 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 [<sup>32</sup>P]-ATP incorporation into immunoprecipitated RyR2 (to ensure that we only measured [<sup>32</sup>P]-ATP incorporation into RyR2 monomers). The <sup>32</sup>P/RyR2 ratio was calculated by dividing <sup>32</sup>P-phosphorylation by the amount of high-affinity [<sup>3</sup>H]-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 [<sup>3</sup>H]-ryanodine binding, 100  $\mu\text{g}$  of protein were incubated with different concentrations of [<sup>3</sup>H]-ryanodine in 0.5 mL of binding buffer (170 mmol/L KCl, 20  $\mu\text{mol/L}$   $\text{CaCl}_2$ , 10 mmol/L MOPS, pH 7.0 for 4 hours at 30°C). For nonspecific binding, 2 mmol/L nonradioactive ryanodine was added. After incubation, the reaction mixture was filtered through Whatman filters and washed with ice-cold buffer (170 mmol/L KCl, 10 mmol/L MOPS).  $B_{\text{max}}$  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.<sup>5</sup> The *trans* chamber (1.0 mL) representing the intra-SR 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 mmol/L): *trans* HEPES 250 and  $\text{Ca}(\text{OH})_2$  53, pH 7.35; *cis* HEPES 250, Tris 125, EGTA 1.0, and  $\text{CaCl}_2$  0.5, pH 7.35. Free  $[\text{Ca}^{2+}]$  was calculated by CHELATOR software.<sup>16</sup> At the conclusion of each experiment, ryanodine (5  $\mu\text{mol/L}$ ) or ruthenium red (20  $\mu\text{mol/L}$ ) 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 U/kg IV). The heart was immediately excised, rinsed twice in ice-cold, Krebs-Henseleit (KH) solution (in mmol/L: NaCl 137, KCl 5.4, HEPES 20,  $\text{MgSO}_4$  1.2,  $\text{NaH}_2\text{PO}_4$  1.2, glucose 15,  $\text{CaCl}_2$  2.0, and NaF 1.0, saturated with  $\text{O}_2$ , pH 7.4), and perfused by a modified Langendorff 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  $\mu\text{mol/L}$ ; Calbiochem), or W-7 (0.1  $\mu\text{mol/L}$ , Biomol Research Laboratories) or (2) the nonselective  $\beta$ -adrenergic receptor ( $\beta$ -AR) agonist isoproterenol (ISO; 0.1  $\mu\text{mol/L}$ ). At completion of each experiment, the ventricular myocardium was flash frozen, and stored at  $-80^\circ\text{C}$ .

### Heart Failure Model

Twenty Sprague-Dawley rats (300 to 400 g; Harlan, Indianapolis, Ind) underwent left coronary artery ligation via left thoracotomy as previously described,<sup>17</sup> 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.

## Data Analysis and Statistics

Data are presented as mean  $\pm$  SEM. The unpaired Student's *t* test was used for statistical comparison of mean values between two experimental groups, 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 Serine<sup>2815</sup> on RyR2

It has been suggested that PKA and CaMKII both phosphorylate the same residue on RyR2 (Ser<sup>2809</sup>),<sup>3,7</sup> (Figure 1A). We have previously shown that substituting an alanine for Ser<sup>2809</sup> 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 Ser<sup>2809</sup> is the unique PKA site on the channel.<sup>4</sup> 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.<sup>18</sup> The peptide used to identify the CaMKII site by Jones and colleagues<sup>7</sup> contains, in addition to Ser<sup>2809</sup>, one potential CaMKII site, S<sup>2815</sup>-V-D (Figure 1A), and a second potential CaMKII site is located at Ser<sup>2835</sup>. We found that GST-fusion protein mutant RyR2-S2815A channels could not be phosphorylated by CaMKII, whereas the RyR2-S2835A mutation was able to be CaMKII phosphorylated (Figure 1B). In order to determine whether Ser<sup>2815</sup> was the unique CaMKII 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 Ser<sup>2815</sup> 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 Ser<sup>2809</sup>, which is part of the fight-or-flight response, dissociates the regulatory subunit FKBP12.6 from the RyR2 macromolecular complex.<sup>4</sup> RyR2-S2809D channels mimic constitutively PKA 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- $\delta$  isoform was a component of the RyR2 macromolecular complex, consistent with earlier reports that CaMKII- $\delta$  copurifies with RyR2<sup>10</sup> (Figure 1D). To

demonstrate that the CaMKII in the RyR2 complex was active, we added Ca<sup>2+</sup> 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.

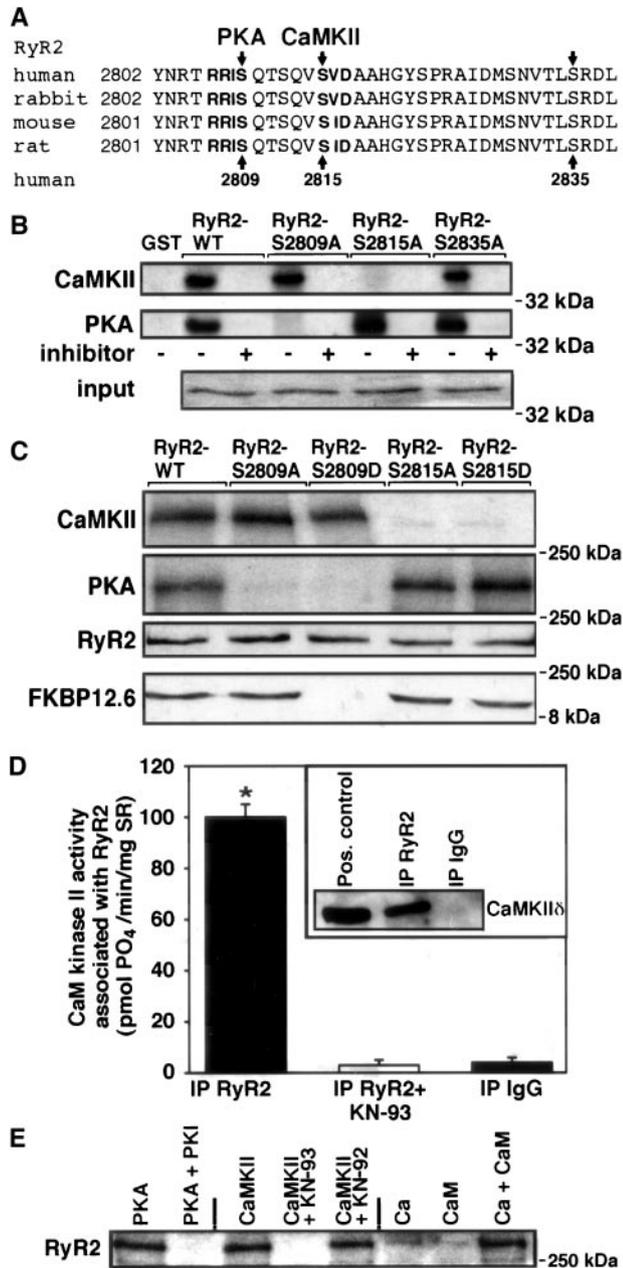
### CaMKII Phosphorylation of RyR2 Increases Open Probability

Studies examining the functional effects of CaMKII phosphorylation of RyR2 have yielded conflicting results.<sup>7-9,19,20</sup> 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 (Ser<sup>2809</sup>) did not increase open probability ( $P_o$ ) compared with RyR2-WT, whereas PKA phosphorylated RyR2-S2815A channels did show increased  $P_o$  and subconductance levels (Figure 2B). RyR2-S2809D channels, which mimic constitutively PKA phosphorylated RyR2, displayed increased  $P_o$ , similar to PKA phosphorylated RyR2-WT (or RyR2-S2815A) channels. In contrast, CaMKII phosphorylation of RyR2-S2815A channel did not increase  $P_o$ , whereas RyR2-S2809A channels could be activated by CaMKII (Figure 2B, bottom). RyR2-S2815D channels, mimicking constitutively CaMKII phosphorylated RyR2, displayed increased  $P_o$ , 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 Ca<sup>2+</sup>-dependent activation at [Ca<sup>2+</sup>] 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 Ca<sup>2+</sup> signals, we sought to define the effects of CaMKII on RyR2 because RyR2 is a Ca<sup>2+</sup> 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  $\approx$ 100 bpm and paced (HR 200 bpm) hearts. The



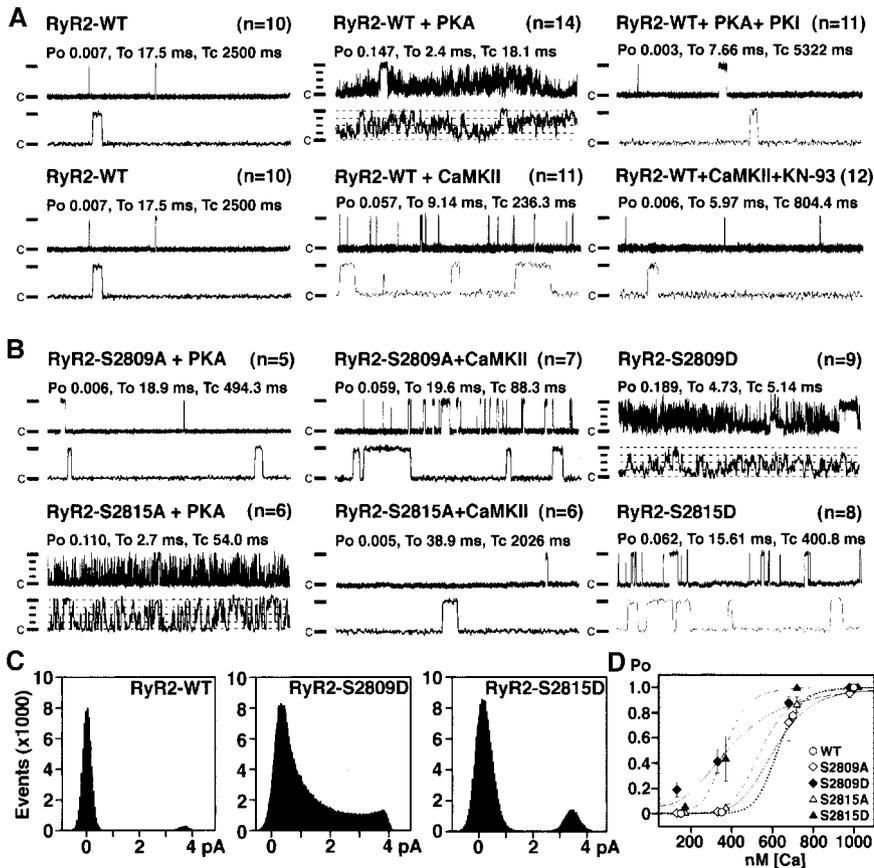
**Figure 1.** CaMKII phosphorylates RyR2 at Ser<sup>2815</sup>. **A**, Sequence alignment of RyR2 from different species showing that the S-x-D CaMKII consensus site is evolutionary conserved from mouse to human in RyR2. **B**, GST-fusion proteins containing amino acids 2777 to 2866 of the human ryanodine receptor 2 (RyR2) were backphosphorylated with PKA or CaMKII, in the presence or absence of specific inhibitors PKI<sub>5-24</sub> or KN-93, respectively. Alanine substitution of Ser<sup>2809</sup> prevents PKA phosphorylation of RyR2, whereas alanine substitution of Ser<sup>2815</sup> prevents CaMKII phosphorylation. Coomassie staining (input) showing GST-fusion protein input. **C**, Full-length human wild-type RyR2 or mutant RyR2 channels, coexpressed with FKBP12.6, were phosphorylated with PKA or CaMKII. Mutant RyR2-S2809A channels cannot be PKA phosphorylated, whereas the mutant RyR2-S2815A cannot be CaMKII phosphorylated. RyR2 immunoblot shows equal loading of protein samples. FKBP12.6 immunoblot shows that RyR2-S2809D, mimicking constitutively PKA phosphorylated RyR2, did not bind FKBP12.6. RyR2-S2815D, mimicking CaMKII phosphorylated RyR2, does bind FKBP12.6. **D**, Human cardiac SR (50 μg) was immunoprecipitated with anti-RyR2 antibody before the measurement of CaMKII activity. Bar graph depicts the amount of radioac-

activity of RyR2 channels was increased at the higher heart rate as manifested by an increase in the open probability ( $P_o$ ) measured at 150 nmol/L  $[Ca^{2+}]_i$  ( $0.089 \pm 0.018$ ;  $n=10$ ), compared with nonpaced ( $0.007 \pm 0.003$ ;  $n=11$ ) (Figure 3B). Moreover, this increase in RyR2 activity was also blocked by CaMKII inhibitors KN-93 ( $0.013 \pm 0.003$ ;  $n=12$ ) or W-7 ( $0.019 \pm 0.003$ ;  $n=10$ ) (Figure 3B). The CaMKII-dependent effect resulted in increased sensitivity of RyR2 channels from paced rabbit hearts to activation by  $Ca^{2+}$ , compared with control (Figure 3C). In contrast,  $Ca^{2+}$  sensitivity of RyR2 from KN-93-treated paced hearts was not increased (Figure 3C). Taken together, these data suggest that in response to increased heart rates, RyR2 is activated due to increased sensitivity to activation by  $Ca^{2+}$ , and that this effect depends on CaMKII.

We next wanted to determine whether or not the CaMKII-dependent activation of RyR2 at increased heart rates was associated with CaMKII phosphorylation of the channel itself. Using coimmunoprecipitations, we showed that CaMKII is part of the RyR2 macromolecular complex (Figures 3D, 1D, and 1E). Moreover, the amount of CaMKII bound to RyR2 did not change at increased heart rates (Figures 3D and 3E). However, increasing the heart rate from 100 to 200 bpm resulted in a  $\approx 2$ -fold increase in CaMKII autophosphorylation at Thr<sup>287</sup> consistent with activation of CaMKII in the RyR2 macromolecular complex (Figures 3D and 3E). In addition, there was an increased amount of calmodulin (CaM) in the RyR2 macromolecular complex (Figures 3D and 3E) at higher heart rates consistent with previous studies showing that autophosphorylation of CaMKII leads to an increase in the affinity of CaM for CaMKII.<sup>21</sup> Concomitant with the increase in CaMKII activity in the RyR2 complex there was a  $\approx 2$ -fold increase in CaMKII phosphorylation of RyR2 (Figures 3F and 3G) detected using a phosphoepitope-specific antibody that recognizes CaMKII phosphorylated Ser<sup>2815</sup> on RyR2 (Figures 3A through 3C). CaMKII inhibitors KN-93 (Figures 3F and 3G) and W-7 (data not shown) inhibited CaMKII phosphorylation of RyR2.

In order to investigate whether CaMKII activation was independent from the  $\beta$ -adrenergic signaling pathway (eg, the fight-or-flight response), rabbit hearts were perfused with 0.1 μmol/L isoproterenol, which increased PKA but not CaMKII phosphorylation of RyR2 (Figures 3F and 3G). In contrast to PKA phosphorylation, CaMKII phosphorylation of RyR2 does not dissociate FKBP12.6 from the RyR2 macromolecular complex (Figures 3F and 3G). Thus, increased heart rate is associated with CaMKII activation and increased CaMKII phosphorylation of RyR2 on Ser<sup>2815</sup>, which is distinct from the PKA site on RyR2 (Ser<sup>2809</sup>). Moreover, this increase in CaMKII phosphorylation of RyR2 at elevated heart rates was associated with

diverse phosphate incorporated into a CaMKII substrate peptide in the presence of PKA and PKC inhibitors, indicating that CaMKII activity is present in the RyR2 macromolecular complex. Negative controls included an immunoprecipitation with IgG, and addition of the specific CaMKII inhibitor (KN-93). Insert shows a CaMKII- $\delta$  Western blot after an immunoprecipitation with anti-RyR2. \* $P < 0.01$ . **E**, In the absence of exogenous CaMKII, addition of  $Ca^{2+}$  and CaM-enhanced CaMKII phosphorylation of immunoprecipitated RyR2.



**Figure 2.** CaMKII phosphorylation activates RyR2. **A**, Single-channel recordings of RyR2-WT and mutant RyR2, untreated or treated with PKA in the absence or presence of inhibitor PKI<sub>5-24</sub> (top) or with CaMKII in the absence or presence of inhibitor KN-93 (bottom). CaMKII phosphorylation of RyR2 increases open probability ( $P_o$ ); PKA phosphorylation also increases  $P_o$  and induces subconductance states. Parameters  $P_o$ ,  $T_o$  (mean open time), and  $T_c$  (mean closed time) are presented for the shown single-channel experiment. All channel traces shown in **A** through **C** were recorded at 150 nmol/L  $[Ca^{2+}]$ . **B**, Mutant channel RyR2-S2809A was unable to be activated by PKA but retained CaMKII-induced activation. In contrast, that mutant RyR2-S2815A could not be activated by CaMKII but retained intact PKA phosphorylation induced activation. Mutant RyR2-S2815D channels functionally mimicked CaMKII phosphorylated RyR2. **C**, Representative amplitude histograms revealing increased open probability of RyR2-S2809D and RyR2-S2815D channels. RyR2-S2809D channels exhibited subconductance openings, consistent with channels depleted of FKBP12.6 (see Figure 3C). In contrast, RyR2-S2815D channels did not show subconductance openings. **D**, Open probability ( $P_o$ ) versus cytoplasmic  $[Ca^{2+}]$  showing that CaMKII phosphorylation simulated by the mutant RyR2-S2815D channel results in increased  $Ca^{2+}$  sensitivity of RyR2 channels. Channel openings are upward, the dash indicates the level of full openings (4 pA); c indicates the closed state. Channels are shown at 5 seconds (left tracing) and 500 ms (right tracing) time scales.

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 Thr<sup>17</sup>, in the absence of PLB-Ser<sup>16</sup> phosphorylation by PKA, suggesting that activation of CaMKII is part of a distinct signaling pathway triggered by increased heart rate.<sup>22</sup> Therefore, we assessed the phosphorylation status of PLB in response to an increased heart rate in the rabbit Langendorff model. In contrast to PLB-Ser<sup>16</sup> phosphorylation, PLB-Thr<sup>17</sup> 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

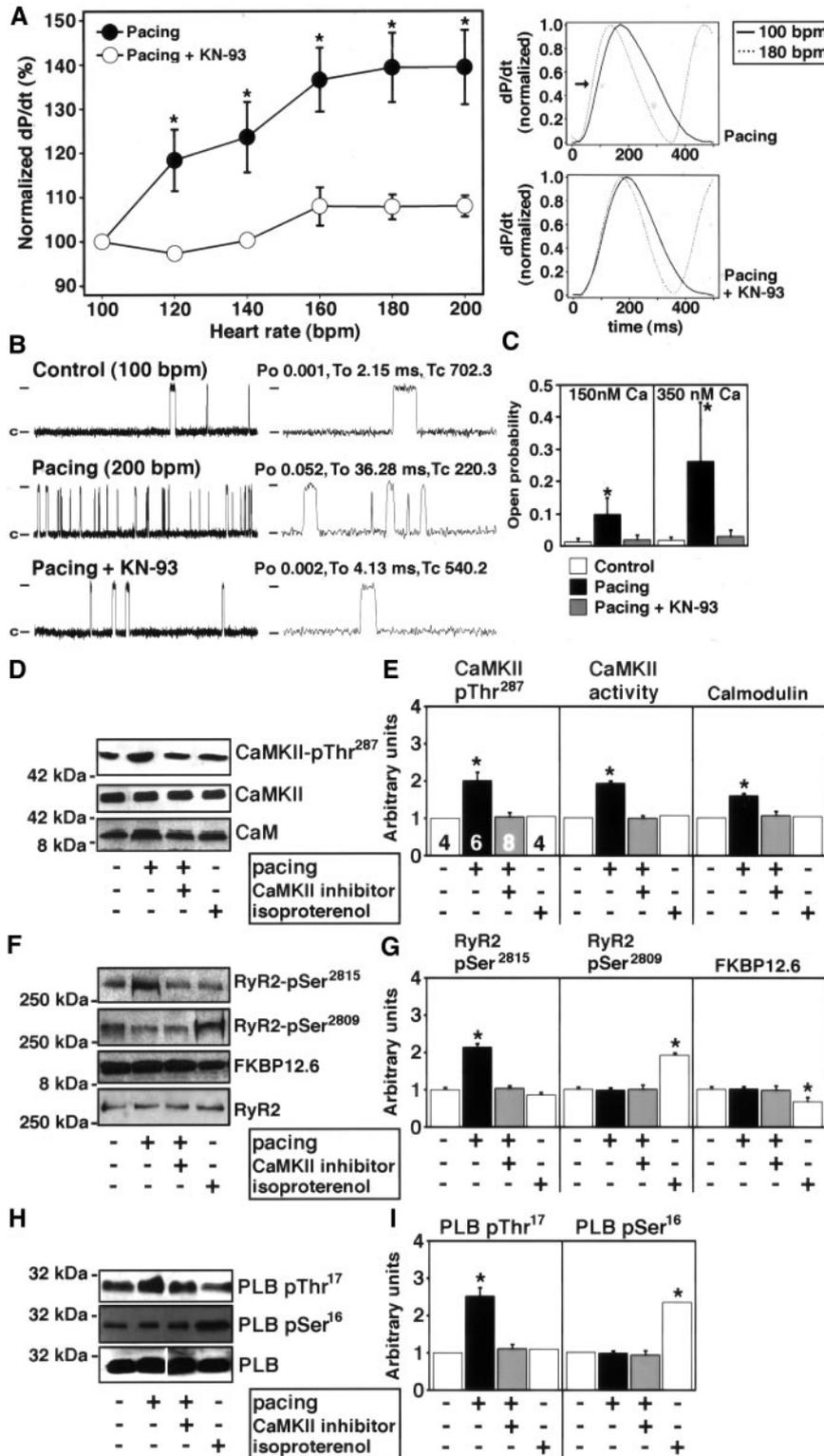
Previous studies have reported that CaMKII may phosphorylate more than one CaMKII site on RyR2.<sup>9,23–26</sup> Rabbit cardiac SR membranes were first dephosphorylated using alkaline phosphatase (Figure 4A). Complete dephosphorylation was confirmed using an antibody that specifically recognizes the dephosphorylated RyR2-Ser<sup>2809</sup>. 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 <sup>32</sup>P-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  $Ca^{2+}$  and calmodulin (CaM) (Figure 4A).

In order to determine the stoichiometry of PKA and CaMKII phosphorylation of RyR2, the amount of RyR2 in 100  $\mu$ g of SR was quantified using [<sup>3</sup>H]-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 <sup>32</sup>P-ATP incorporated into immunoprecipitated RyR2 indicated that PKA incorporates  $1.00 \pm 0.18$  mol of phosphate per mol of RyR2 monomer, whereas CaMKII incorporates  $1.50 \pm 0.07$  mol of phosphate per mol of RyR2 monomer (Figure 4B). Pretreatment with nonradioactive PKA did not change the amount of <sup>32</sup>P-ATP incorporated into RyR2 by CaMKII ( $1.51 \pm 0.01$ ).

### 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 of stimulation for reasons that are not well understood.<sup>27</sup> In this study, we showed that in control (sham-operated; n=8) animals the level of CaMKII phosphorylation of RyR2-Ser<sup>2815</sup> increased proportionately with elevated heart rate ( $r=0.905$ ; Figure 5A). However, in rats with heart failure after myocardial infarction (n=20), the relation-



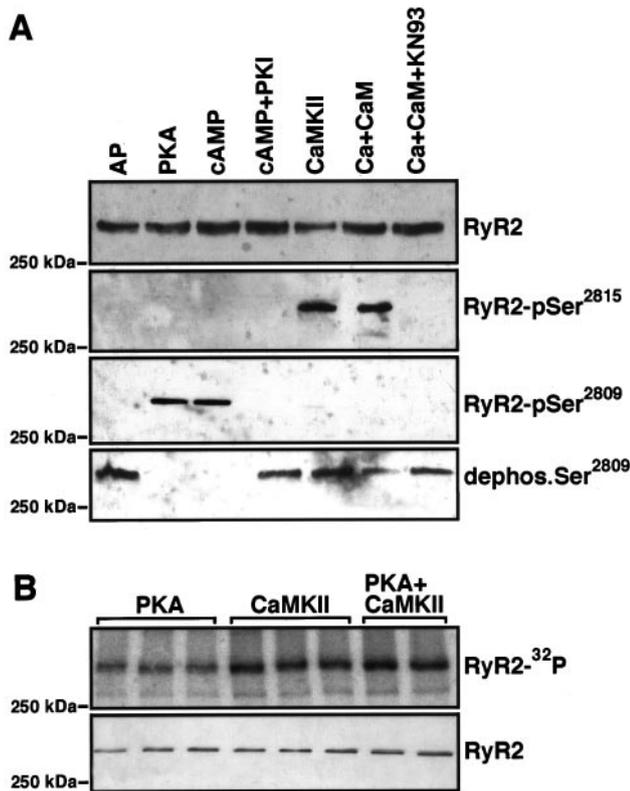
**Figure 3.** Increasing heart rate associated with CaMKII phosphorylation of cardiac ryanodine receptors. **A**, Increased heart rate was associated with enhanced myocardial contractility (dP/dt) in Langendorff-perfused rabbit hearts (closed circles; n=6), and the CaMKII inhibitor KN-93 blocked this effect (open circles; n=4). \*P<0.05. Panels at right show that both Ca<sup>2+</sup> release and reuptake were accelerated at higher heart rates and that the CaMKII inhibitor KN-93 inhibited acceleration of SR Ca<sup>2+</sup> release but not acceleration of Ca<sup>2+</sup> reuptake. **B**, Single-channel tracings at 150 nmol/L *cis* [Ca<sup>2+</sup>] showing increased RyR2 P<sub>o</sub> after pacing (to 200 bpm; n=11), as compared with nonpaced hearts (100 bpm; n=10). In these experiments, hearts were paced and then flash frozen and channels isolated in the presence of phosphatase inhibitors. P<sub>o</sub> did not increase with pacing in the presence of CaMKII inhibitor KN-93 (n=12). Channel openings are upward; the dash indicates the level of full openings (4 pA); and c indicates the closed state. Channels are shown at 5 seconds (left tracing) and 500 ms (right tracing). **C**, Rapid pacing increased the Ca<sup>2+</sup> sensitivity of RyR2 channels, which was prevented by the CaMKII inhibitor KN-93. **D** and **E**, After an increase in heart rate, CaMKII activity and autophosphorylation of CaMKII at Thr<sup>287</sup> significantly increased (\*P<0.05, effects that were blocked by CaMKII inhibitors KN-93 and W-7; data not shown). Consistent with increased autophosphorylation of CaMKII, increased amounts of CaM were coimmunoprecipitated with the RyR2-CaMKII complex. Numbers in bar graphs indicate the number of experiments. **F** and **G**, Increased heart rates induced CaMKII phosphorylation of RyR2, which could be inhibited by CaMKII inhibitors. In contrast, increasing heart rate did not increase PKA phosphorylation of RyR2. CaMKII phosphorylation of RyR2 did not lead to dissociation of FKBP12.6 from the macromolecular channel complex.

ship between elevated heart rate and increased CaMKII phosphorylation of RyR2 was absent (*r*=0.457; Figure 5B).

### Discussion

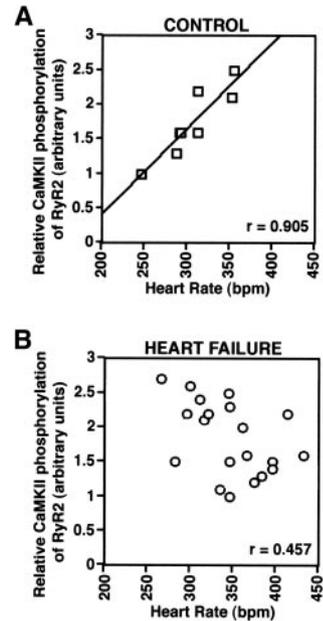
This study identifies the principal CaMKII phosphorylation site on the cardiac ryanodine receptor (RyR2) using site-directed mutagenesis and shows that phosphorylation at this site activates

the channel. We showed that Ser<sup>2815</sup> is the principal CaMKII phosphorylation site on recombinant RyR2, whereas Ser<sup>2809</sup> is the target of phosphorylation by PKA.<sup>4</sup> Previous studies have suggested that Ser<sup>2809</sup> may be the phosphorylation site of both PKA and CaMKII based on sequencing of tryptic phosphopeptides<sup>3,7</sup> or a phosphopeptide-specific antibody,<sup>26</sup> respectively. Our studies are the first to use site-directed mutagenesis of the



**Figure 4.** Stoichiometry of CaMKII phosphorylation of native RyR2. A, Rabbit cardiac SR was treated with alkaline phosphatase to dephosphorylate the RyR2 in the microsomes. Dephosphorylated RyR2s were then phosphorylated by addition of either PKA, cAMP (to activate endogenous PKA), CaMKII, or  $\text{Ca}^{2+}$  and calmodulin (to activate endogenous CaMKII). The specificity of the cAMP- and calmodulin-dependent phosphorylations was demonstrated by the addition of PKI and KN-93, respectively. Proteins were separated by 6% PAGE and probed for antibodies against total RyR2 (5029), RyR2 phosphorylated at Ser<sup>2809</sup> (RyR2-pSer<sup>2809</sup>), RyR2 phosphorylated at Ser<sup>2815</sup> (RyR2-pSer<sup>2815</sup>), and an antibody that specifically recognizes nonphosphorylated RyR2 at Ser<sup>2809</sup>. B, To determine the maximum level of phosphorylation for RyR2, dephosphorylated cardiac SR was immunoprecipitated with 5029 antibody and the immunoprecipitate was phosphorylated with either PKA or CaMKII using  $\gamma\text{P}^{32}\text{-ATP}$ . In some experiments, the immunoprecipitate was prephosphorylated with PKA and unlabeled ATP before phosphorylation with CaMKII and  $\gamma\text{P}^{32}\text{-ATP}$ .

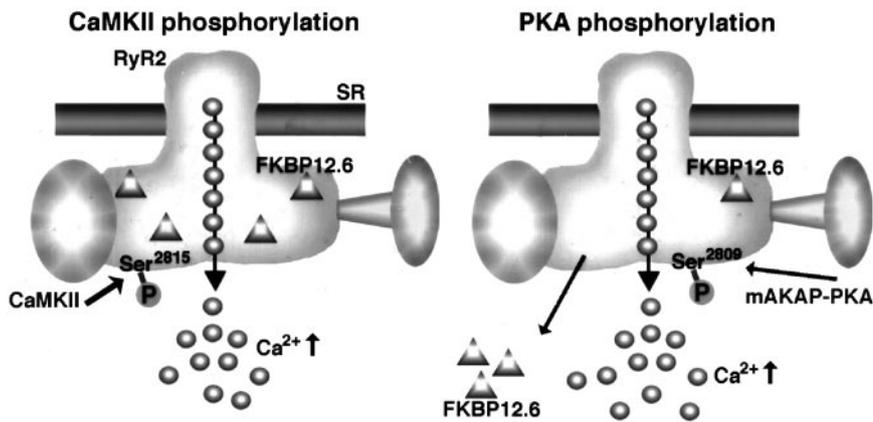
full-length RyR2 channel to show that there are two distinct phosphorylation sites for PKA and CaMKII, because the mutation RyR2-S2809A only prevents PKA phosphorylation, and the mutation RyR2-S2815A only abolishes CaMKII phosphorylation of RyR2, respectively. Moreover, we were also able to show that both serines can be independently phosphorylated by PKA and CaMKII, respectively, using phosphoepitope antibodies specific for either phosphorylated Ser<sup>2809</sup> or Ser<sup>2815</sup> 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.<sup>3,7</sup>



**Figure 5.** Relationship between heart rate and CaMKII phosphorylation of RyR2 in heart failure. A, In sham-operated rats, there is a significant correlation between increased heart rate (HR) and CaMKII phosphorylation of RyR2, as detected using a phosphoepitope RyR2-pSer<sup>2815</sup> antibody (n=8). B, In heart failure rats, there was no correlation between HR and CaMKII phosphorylation of RyR2 (n=20).

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.<sup>9,23–25</sup> In contrast, Rodriguez et al<sup>26</sup> 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 [<sup>3</sup>H]-ryanodine, but rather normalized the amount of <sup>32</sup>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 al<sup>28</sup> have previously reported that under similar conditions stoichiometric PKA phosphorylation may take at least 3 minutes. Thus, our results suggest that besides Ser<sup>2815</sup>, a second CaMKII site may be partially phosphorylated in native RyR2 under pharmacological conditions, whereas in recombinant RyR2 only Ser<sup>2815</sup> 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.

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 Ser<sup>2809</sup>. Previous studies have suggested that CaMKII may be part of the RyR2 macromolecular complex based on copurification<sup>25,29,30</sup> and the presence of endogenous CaMKII activity associated with purified RyR2.<sup>25,30</sup> In the present study,



**Figure 6.** Model of regulation of RyR2 by CaMKII and PKA phosphorylation. RyR2 is regulated by 2 distinct phosphorylation-dependent pathways activating SR  $\text{Ca}^{2+}$  release via RyR2: one is activated by increased heart rate and involves CaMKII phosphorylation of RyR2 at Ser<sup>2815</sup> (left); the other is activated by the sympathetic nervous system as part of the fight-or-flight stress response and involves  $\beta$ -adrenergic receptor/PKA phosphorylation of RyR2 at Ser<sup>2809</sup> (right). Protein kinase A (PKA) is bound to RyR2 through the adaptor protein mAKAP. In contrast to PKA phosphorylation, CaMKII phosphorylation of RyR2 does not cause dissociation of FKBP12.6 from the RyR2 macromolecular complex.

we show that CaMKII is indeed part of the RyR2 macromolecular complex using coimmunoprecipitations, in agreement with recent studies by Zhang et al.<sup>10</sup>

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.<sup>4,5,24,31,32</sup> However, several laboratories have challenged the idea that PKA phosphorylation leads to increased channel activity<sup>33,34</sup> and FKBP12.6 release.<sup>33–35</sup> Li et al<sup>33</sup> 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  release during the early phase of EC coupling,<sup>36</sup> which is entirely consistent with our model.<sup>4</sup>

Terentyev et al<sup>37</sup> reported that protein phosphatase 1 (PP1) may increase RyR2 activity. These results, however, contrast with several other studies that reported that PP1 decreases RyR activity<sup>38–43</sup> and that PKA phosphorylation increases RyR activity.<sup>4</sup>

We have previously shown that PKA phosphorylation results in the dissociation of FKBP12.6 from the macromolecular complex.<sup>1,4</sup> In contrast, other groups have shown that FKBP12.6 may bind to PKA phosphorylated RyR2 under certain experimental conditions.<sup>28,34</sup> 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 al<sup>34</sup> and Xiao et al,<sup>44</sup> or added in excess amounts as in Xiao et al<sup>44</sup>), FKBP12.6 may still be able to bind to PKA phosphorylated RyR2 or RyR2-S2809D because it overwhelms the shift in  $K_d$  induced by PKA phosphorylation of Ser<sup>2809</sup>.<sup>45</sup> 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.<sup>5,45</sup> 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.<sup>34,44</sup>

Contradictory results have also been reported regarding the functional effects of CaMKII phosphorylation of RyR2 with some studies showing activation<sup>7–9</sup> and some inactivation.<sup>19,20</sup> Witcher et al<sup>7</sup> 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 al<sup>46</sup> demonstrated that cardiomyocytes isolated from mice overexpressing cytoplasmic CaMKII $\delta_c$  display an increased  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  release.<sup>46,47</sup>

Contractility increases with increasing heart rate, known as the Bowditch phenomenon or positive force-frequency (staircase) relationship.<sup>48,49</sup> The positive force-frequency relationship may require alterations of both intracellular  $\text{Ca}^{2+}$  release and reuptake into the SR, to provide sufficient time for diastolic filling of the ventricle at higher heart rates.<sup>50</sup> An increase in the frequency of intracellular  $\text{Ca}^{2+}$  concentration elevations during increased heart rate activates CaMKII,<sup>51</sup> which is thought to mediate the increased  $\text{Ca}^{2+}$  reuptake into the SR.<sup>52</sup> It has been proposed that speeding of  $\text{Ca}^{2+}$  reuptake is mediated by  $\text{Ca}^{2+}$ /calmodulin-dependent kinase II (CaMKII) phosphorylation of phospholamban (PLB) at Thr<sup>17</sup>, which would increase SERCA2a activity.<sup>22,53</sup> 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,<sup>50,54</sup> suggesting additional mechanisms may contribute to enhanced intracellular  $\text{Ca}^{2+}$  reuptake at faster heart rates.

Although some studies have suggested that Ser<sup>16</sup>-phosphorylation of phospholamban (PLB) precedes or is required for Thr<sup>17</sup>-PLB phosphorylation,<sup>55,56</sup> other studies have provided compelling evidence that PLB can be phosphorylated at Thr<sup>17</sup>

by CaMKII, independently of prior phosphorylation at Ser<sup>16</sup> by PKA.<sup>22,57</sup> 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  $\beta$ -AR stimulation or increases in heart rate, respectively.

Shortening of the Ca<sup>2+</sup> transient at faster heart rates may also be caused by a decrease in time to peak [Ca<sup>2+</sup>], which may reflect an enhanced Ca<sup>2+</sup> sensitivity of the SR Ca<sup>2+</sup> release mechanism.<sup>58</sup> 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.<sup>3</sup> Our data suggest that at increased heart rates, the increase in time-averaged [Ca<sup>2+</sup>], leads to activation of CaMKII associated with RyR2.<sup>51</sup> The increased Ca<sup>2+</sup> sensitivity of CaMKII-phosphorylated RyR2 may lead to a faster (and larger) upstroke of the Ca<sup>2+</sup> transient.<sup>12,46</sup> 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-Ser<sup>2815</sup> could contribute to the loss of the force-frequency relationship in heart failure.<sup>27</sup>

The present study suggests that two distinct phosphorylation-dependent pathways are available for activating RyR2: one is activated by increased heart rate and involves Ca<sup>2+</sup>/CaMKII-dependent phosphorylation of RyR2 at Ser<sup>2815</sup>; the other is activated by the sympathetic nervous system as part of the fight-or-flight stress response and involves  $\beta$ -adrenergic receptor/PKA phosphorylation of RyR2 at Ser<sup>2809</sup> (Figure 6).<sup>4</sup> 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 gating<sup>59</sup> and an increased propensity for cardiac arrhythmias.<sup>5</sup>

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