Protein Kinase A Phosphorylation at Serine-2808 of the Cardiac Ca\(^{2+}\)-Release Channel (Ryanodine Receptor) Does Not Dissociate 12.6-kDa FK506-Binding Protein (FKBP12.6)

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Abstract—Dissociation of FKBP12.6 from the cardiac Ca\(^{2+}\)-release channel (RyR2) as a consequence of protein kinase A (PKA) hyperphosphorylation of RyR2 at a single amino acid residue, serine-2808, has been proposed as an important mechanism underlying cardiac dysfunction in heart failure. However, the issue of whether PKA phosphorylation of RyR2 can dissociate FKBP12.6 from RyR2 is controversial. To additionally address this issue, we investigated the effect of PKA phosphorylation and mutations at serine-2808 of RyR2 on recombinant or native FKBP12.6-RyR2 interaction. Site-specific antibodies, which recognize the serine-2808 phosphorylated or nonphosphorylated form of RyR2, were used to unambiguously correlate the phosphorylation state of RyR2 at serine-2808 with its ability to bind FKBP12.6. We found that FKBP12.6 can bind to both the serine-2808 phosphorylated and nonphosphorylated forms of RyR2. The S2808D mutant thought to mimic constitutive phosphorylation also retained the ability to bind FKBP12.6. Complete phosphorylation at serine-2808 by exogenous PKA disrupted neither the recombinant nor native FKBP12.6-RyR2 complex. Furthermore, binding of site-specific antibodies to the serine-2808 phosphorylation site did not dissociate FKBP12.6 from or prevent FKBP12.6 from binding to RyR2. Taken together, our results do not support the notion that PKA phosphorylation at serine-2808 dissociates FKBP12.6 from RyR2. (Circ Res. 2004;94:487-495.)

Key Words: heart failure • ryanodine receptor • protein kinase A phosphorylation • FKBP12.6 binding • phospho-specific antibody

Ryanodine receptors (RyRs) are a family of intracellular Ca\(^{2+}\)-release channels located in the sarcoplasmic reticulum of a variety of cells. They play an essential role in various cellular functions, and genetic defects in the RyR genes have been linked to diseases.1–4 For instance, mutations in the cardiac RyR (RyR2) gene are associated with cardiac arrhythmias and sudden death.5 In addition to these genetic defects, abnormal function of RyRs as a result of defective phosphorylation has also been attributed to the pathogenesis of diseases such as heart failure.6

In failing hearts, it has been shown that RyR2 was hyperphosphorylated at serine-2808 (corresponding to serine-2809 in rabbit RyR2), which was originally identified as a unique phosphorylation site for the Ca\(^{2+}\) and calmodulin-dependent protein kinase II (CaMKII),7 by the cAMP-dependent protein kinase (PKA).8 Alterations in the interaction between RyR2 and the 12.6-kDa FK506-binding protein (FKBP12.6) were also observed in heart failure. The level of FKBP12.6 bound to RyR2 or associated with sarcoplasmic reticulum (SR) vesicles9 was significantly reduced in several models of heart failure. Investigations of single RyR2 channels from failing hearts in lipid bilayers have revealed an enhanced sensitivity of the channel to Ca\(^{2+}\) activation and altered gating and conduction.8 Parallel to these observations, phosphorylation of RyR2 from normal hearts by exogenous PKA caused dissociation of FKBP12.6 from RyR2 and alterations in channel activation, gating, and conduction.8 Furthermore, a single mutation of serine-2808 to aspartate (S2808D) abolished FKBP12.6 binding to RyR2.10 These observations have led to the notions that FKBP12.6 cannot bind to serine-2808 phosphorylated RyR2 and that PKA phosphorylation physiologically regulates FKBP12.6-RyR2 interaction.8,10 As a result, alteration in FKBP12.6-RyR2 interaction attributable to PKA hyperphosphorylation was put forward as a novel mechanism for cardiac dysfunction in heart failure and exercise-induced sudden cardiac death.8,10

A central feature of the theory proposed by Marks et al4 for heart failure is the idea that PKA phosphorylation at a single site, serine-2808, in RyR2 induces dissociation of FKBP12.6. However, this idea is apparently inconsistent with the observation that only PKA phosphorylation, but not phosphorylation by CaMKII, is able to dissociate FKBP12.6 from RyR2,8 despite the fact that both PKA and CaMKII are able to fully phosphorylate serine-2808.11 This raises the question of...
whether phosphorylation at the serine-2808 site is in fact responsible for FKBP12.6 dissociation. Whether PKA phosphorylation can actually dissociate FKBP12.6 from RyR2 has recently been questioned by Jiang et al., who failed to observe dissociation of FKBP12.6 from cardiac microsomal membranes after PKA phosphorylation. Consequently, the issue of FKBP12.6 dissociation by PKA is controversial and requires additional investigations.

Other key aspects of the proposed scheme for cardiac dysfunction in heart failure by Marks et al. were also contested recently in several studies. Jiang et al. showed that the phosphorylation level and basal activity of the RyR2 channel from failing hearts were indistinguishable from those of RyR2 from normal hearts. In the study by Li et al., PKA phosphorylation of RyR2 was found to have no effect on \( \text{Ca}^{2+} \) sparks in mouse cardiac myocytes. Furthermore, Terentyev et al. reported that treatment of permeabilized rat cardiac myocytes with protein phosphatases activated RyR2 and depleted SR \( \text{Ca}^{2+} \) stores, suggesting that dephosphorylation rather than phosphorylation enhances the channel activity of RyR2. The exact molecular mechanism by which FKBP12.6 exerts its effect on RyR2 is also unclear. On the one hand, removal of FKBP12.6 from single RyR2 channels was shown to alter channel conductance, gating, and sensitivity to activation by \( \text{Ca}^{2+} \). But on the other hand, depletion of FKBP12.6 did not seem to significantly affect the single channel properties of RyR2. Therefore, the issues of whether RyR2 is PKA hyperphosphorylated in heart failure, whether PKA phosphorylation increases the sensitivity of the RyR2 channel to \( \text{Ca}^{2+} \) activation, and whether dissociation of FKBP12.6 affects single RyR2 channel properties remain unresolved.

In the present study, we have focused on investigations of whether FKBP12.6 can bind to serine-2808–phosphorylated RyR2 and the S2808D mutant and whether PKA phosphorylation at serine-2808 can dissociate FKBP12.6 from RyR2. Using site-specific antibodies that specifically recognize the phosphorylated and nonphosphorylated serine-2808 site, respectively, we were able to unambiguously determine the phosphorylation state of RyR2 at serine-2808. We have demonstrated that FKBP12.6 is able to interact with both the serine-2808 phosphorylated and nonphosphorylated forms of RyR2 and with the S2808D mutant. Furthermore, we have shown that stoichiometric phosphorylation by exogenous PKA at serine-2808 does not dissociate FKBP12.6 from either reconstituted or native RyR2 channels. These results provide no evidence that PKA phosphorylation of the RyR2 channel at serine-2808 dissociates FKBP12.6 from the channel.

Materials and Methods

The anti–serine-2808 phosphorylation site antiserum was custom-generated by Global Peptide Services (Fort Collins, Colo) in rabbits. The serum was first absorbed by the nonphosphorylated serine-2808 peptide and then purified using the serine-2808 (PO3) peptide conjugated to SulfoLink Coupling Gel as the affinity ligand. Mutations S2808A and S2808D were introduced into the mouse RyR2 by the overlap extension method. Preparation of cell lysate from transfected HEK293 cells and of microsomal membranes from canine cardiac muscle was carried out as described previously. Analysis of FKBP12.6 interaction with RyR2 or mutants S2808A and S2808D was performed by coimmunoprecipitation assay using HEK293 cell lysate or solubilized canine heart microsome. Anti–RyR(34c) was used for immunoprecipitation and detection of RyR2, S2808A, and S2808D. Anti–S2808(PO3) and anti–S2808(deP) antibodies were used to assess the phosphorylation status of RyR2. Anti–FKBP12.6 antibody was used to detect the bound FKBP12.6. PKA phosphorylation and PP1 dephosphorylation of anti–RyR(34c)-immunoprecipitated RyR2 were carried out using 10 \( \mu \)g/mL of the PKA catalytic subunit and 1.0 \( \mu \)g of protein phosphatase PP1, respectively.

A detailed Materials and Methods section can be found in the online data supplement available at http://circres.ahajournals.org.

Results

Generation and Characterization of a Phospho-Specific Antibody Against the Serine-2808 Phosphorylation Site of the Mouse Cardiac Ryanodine Receptor

To be able to correlate the phosphorylation state of RyR2 at serine-2808 with its ability to bind FKBP12.6, we generated
a phosho-specific antibody against the serine-2808 phosphorylation site (Figure 1A). The specificity of the affinity-purified anti-phospho serine-2808 antibody, anti-S2808(PO3), was confirmed by immunoblotting. As shown in Figure 1B, the anti-S2808(PO3) antibody strongly recognizes the phosphorylated peptide, S2808(PO3)-peptide (Figure 1Ba), but not the nonphosphorylated peptide, S2808-peptide (Figure 1Bb). To additionally demonstrate the phospho-dependence of the anti-S2808(PO3) antibody, the S2808-peptide was phosphorylated by PKA and immunoblotted with the antibody. Figure 1Bc shows that the PKA-treated S2808-peptide was then recognized by the anti-S2808(PO3) antibody. These data indicate that the affinity-purified anti-S2808(PO3) antibody is specific to the phosphorylated form of the S2808-peptide.

We next examined the specificity of the anti-S2808(PO3) antibody in the context of the intact RyR2 protein and the phosphorylation state at serine-2808 of RyR2 expressed in HEK293 cells. To obtain a negative control for the antibody, we mutated serine-2808 to alanine (S2808A) to completely remove the phosphorylation site. The RyR2 wild-type (wt) and S2808A mutant were expressed in HEK293 cells, and the expressed RyR2 wt and mutant proteins were immunoprecipitated by an anti-RyR antibody, anti-RyR(34c). The immunoprecipitates were then immunoblotted with the anti-RyR(34c) (Figure 1Ca), anti-S2808(PO3) (Figure 1Cb), or anti-S2808(deP) (Figure 1Cc) antibody. The anti-S2808(deP) antibody was generated against a nonphosphorylated serine-2808 peptide of the rabbit RyR2 and has been characterized to be specific to the nonphosphorylated form of RyR2. As seen in Figure 1C, the RyR2 wt and S2808A mutant were expressed in HEK293 cells at an equivalent level (Figure 1Ca). Neither the anti-S2808(PO3) nor the anti-S2808(deP) antibody interacted with the S2808A mutant (Figures 1Cb and 1Cc, lane 2), indicating that the affinity-purified anti-S2808(PO3) antibody and the anti-S2808(deP) antibody are specific to the S2808-peptide in the context of the intact RyR2 protein. Interestingly, the RyR2 wt was recognized by the anti-S2808(PO3) antibody (Figure 1Cb), indicating that the RyR2 wt protein was phosphorylated at serine-2808 in HEK293 cells. The RyR2 wt protein was also recognized by the anti-S2808(PO3) antibody (Figure 1Cc), indicating that not all of the RyR2 proteins expressed in HEK293 cells were phosphorylated at serine-2808. Phosphorylation of the canine RyR2 protein at serine 2809, which corresponds to serine-2808 in mouse and human RyR2, has also been observed before phosphorylation by exogenous kinases. This raises the interesting possibility that a significant portion of the RyR2 channels may be phosphorylated at serine-2808 (or 2809) in the resting state.

**Effect of Phosphorylation and Mutations at Serine-2808 on FKBP12.6-RyR2 Interaction**

To assess the impact of phosphorylation at serine-2808 on FKBP12.6-RyR2 interaction, we used three RyR2 variants, RyR2 wt and the S2808A and S2808D mutants. These RyR2 variants exhibit different phosphorylation states of serine-2808. Because mutant S2808A cannot be phosphorylated at serine-2808, it represents a completely nonphosphorylated state of serine-2808, whereas the RyR2 wt when expressed in HEK293 cells is partially phosphorylated at serine-2808 (Figure 1C). On the other hand, the S2808D mutant, which is thought to mimic phosphorylation, may correspond to a constitutively phosphorylated state of serine-2808. If the phosphorylation state of serine-2808 dictates the interaction between FKBP12.6 and RyR2, RyR2 wt and the S2808A and S2808D mutants would be expected to differ markedly in their ability to interact with FKBP12.6. To test this possibility, HEK293 cells were transfected with the RyR2 wt, S2808A, or S2808D mutant cDNA. The expressed wt and mutant RyR2 proteins were then pulled down using the GST-FKBP12.6 fusion protein after detergent solubilization. As shown in Figure 2A, all three RyR2 variants, RyR2 wt, S2808A, and S2808D, were pulled down by GST-FKBP12.6. Immunoblotting using the anti-S2808(PO3) antibody confirmed that the RyR2 wt was phosphorylated, but the S2808A and S2808D mutants were not. It should be noted that GST alone did not pull down wt (Figure 2) or mutant RyR2 proteins (not shown). Thus, these results demonstrate that GST-FKBP12.6 is able to interact with RyR2 regardless of the phosphorylation state of serine-2808 and with the S2808D mutant.

**FKBP12.6 Is Coimmunoprecipitated With RyR2 wt and the S2808A and S2808D Mutants by an Anti-RyR Antibody**

RyR2 is a tetrameric structure consisting of four subunits with an identical primary sequence. However, because the RyR2 wt expressed in HEK293 cells was partially phosphorylated, the expressed RyR2 wt is likely to be a heterotetramer containing both phosphorylated and nonphosphorylated subunits. In that case, it would have been possible for GST-FKBP12.6 to interact only with the nonphosphorylated RyR2 subunits, and therefore the presence of the phosphorylated RyR2 in the GST-FKBP12.6 precipitates seen in Figure 2 would be a result of coprecipitation with the nonphosphory-
lated subunit by GST-FKBP12.6. To address this possibility, we carried out a reciprocal experiment in which the wt and mutant RyR2 proteins were immunoprecipitated by the anti-RyR(34c) antibody in the presence of exogenous FKBP12.6 or in the presence of FKBP12.6 plus rapamycin, a drug known to disrupt FKBP12.6-RyR2 interaction. We reasoned that if FKBP12.6 interacts only with the serine-2808 nonphosphorylated RyR2 subunits, the amount of FKBP12.6 associated with the partially phosphorylated RyR2 wt would be relatively less than that associated with the completely nonphosphorylated S2808A mutant. However, as seen in Figure 3, all three RyR2 variants, whether phosphorylated or nonphosphorylated at serine-2808, were able to precipitate a similar level of FKBP12.6 (Figure 3B, lanes 1 through 3). The interaction of FKBP12.6 with the wt and mutant RyR2 was sensitive to rapamycin. Binding of FKBP12.6 to both RyR2 wt and the mutants was abolished in the presence of the drug (Figure 3B, lanes 4 through 6). The PP1-treated (lanes 2 and 3) and untreated (lane 1) immunoprecipitates were incubated with exogenous FKBP12.6 (250 nmol/L) with (lane 3) or without rapamycin (5 μmol/L) (lanes 1 and 2). Immunoprecipitates were blotted with the anti-RyR(34c) antibody (A and C), anti-S2808(PO3) antibody (D), or the anti-FKBP12.0/12.6 antibody (B and E). The purified FKBP12.0 and FKBP12.6 proteins (10 to 20 ng) were used as controls. Multiple high-molecular-weight bands detected in B were most likely derived from the immunoprecipitated mouse monoclonal antibody, anti-RyR(34c), that weakly cross-reacted with the anti-rabbit IgG secondary antibody.

Figure 3. Rapamycin-sensitive commu-
noprecipitation of FKBP12.6 with RyR2 wt and the S2808A and S2808D mutants. A and B, Expressed RyR2(wt), S2808A, and S2808D were immunoprecipitated by the anti-RyR(34c) antibody in the presence of exogenous FKBP12.6 (250 nmol/L) (lanes 1 through 3) or in the presence of exogenous FKBP12.6 (250 nmol/L) plus rapamycin (5 μmol/L) (lanes 4 through 6). C through E, Expressed RyR2(wt) was immunoprecipitated by the anti-RyR(34c) antibody and the immunoprecipitate was treated without or with the protein phosphatase PP1. The PP1-treated (lanes 2 and 3) and untreated (lane 1) immunoprecipitates were incubated with exogenous FKBP12.6 (250 nmol/L) with (lane 3) or without rapamycin (5 μmol/L) (lanes 1 and 2). Immunoprecipitates were blotted with the anti-RyR(34c) antibody (A and C), anti-S2808(PO3) antibody (D), or the anti-FKBP12.0/12.6 antibody (B and E). The purified FKBP12.0 and FKBP12.6 proteins (10 to 20 ng) were used as controls. Multiple high-molecular-weight bands detected in B were most likely derived from the immunoprecipitated mouse monoclonal antibody, anti-RyR(34c), that weakly cross-reacted with the anti-rabbit IgG secondary antibody.

RyR2 wt and the S2808A and S2808D Mutants Are Capable of Forming Complexes With FKBP12.6 in HEK293 Cells

It is clear that RyR2 containing either a phosphorylated or nonphosphorylated serine-2808 can form a complex with GST-FKBP12.6 or FKBP12.6 in vitro. To determine whether the RyR2 wt and mutants interact with FKBP12.6 in HEK293 cells, we cotransfected the cells with FKBP12.6 and wt or mutant RyR2 cDNAs. As shown in Figure 4, RyR2 wt, but not the S2808A or S2808D mutant, was phosphorylated at serine-2808. Importantly, all three RyR2 variants, the phosphorylated RyR2 wt, the nonphosphorylated S2808A mutant, and the S2808D mutant, thought to mimic constitutive phosphorylation were able to precipitate the coexpressed FKBP12.6 (Figure 4C, lanes 4 through 6). In another series of experiments, we treated the FKBP12.6-RyR2 complex without or with phosphatase PP1, which completely dephosphorylated serine-2808 (Figure 4B, lane 8). As shown in Figure 4C (lanes 7 and 8), a complete dephosphorylation of serine-2808 did not dissociate FKBP12.6 from RyR2. No FKBP12.6 was detected in the anti-RyR(34c) immunoprecipitates when HEK293 cells were transfected with the RyR2 wt or mutant alone (Figure 4C, lanes 1 through 3) or with FKBP12.6 alone (data not shown). These data additionally demonstrate that FKBP12.6 can interact with both the serine-2808 phosphorylated and nonphosphorylated forms of RyR2.

RyR2 wt and the S2808A and S2808D Mutants Are Capable of Forming Complexes With FKBP12.6 in HEK293 Cells

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Complete Phosphorylation of RyR2 at Serine-2808 by Exogenous PKA Does Not Dissociate FKBP12.6 From RyR2

To additionally investigate whether phosphorylation at serine-2808 has any effect on FKBP12.6 binding to RyR2, RyR2 wt and the S2808A and S2808D mutants were coexpressed with FKBP12.6 in HEK293 cells. The resulting FKBP12.6-RyR2 (wt) complexes were isolated by immunoprecipitation using the anti-RyR(34c) antibody and were phosphorylated by exogenous PKA or by an inactive (boiled) PKA. Immunoblotting with the anti-RyR(34c) antibody revealed a similar level of expression of the RyR2 wt and mutants (Figure 5A). The phosphorylation state at serine-2808 was assessed using the anti-S2808(PO3) and anti-S2809(deP) antibodies. As shown in Figures 5B and 5C, only RyR2 wt was phosphorylated. Treatment with an active PKA resulted in an increase in the anti-S2808(PO3) antibody signal (compare lane 1 with lane 4 in Figure 5B) and the disappearance of the anti-S2808(deP) antibody signal (Figure 5C, lane 1), indicating that serine-2808 had been completely phosphorylated by exogenous PKA. The effect of PKA phosphorylation on the interaction between FKBP12.6 and the RyR2 wt or mutants was then examined by immunoblotting with the anti-FKBP12.0/12.6 antibody. As shown in Figure 5D, no FKBP12.6 was detected in the supernatant fractions of immunoprecipitates treated with an active or inactive PKA, whereas strong FKBP12.6 signals were detected in the pellet fractions. Thus, PKA phosphorylation of RyR2 at serine-2808 to a full stoichiometry does not dissociate FKBP12.6.

Antibody Binding to the Serine-2808 Phosphorylation Site Does Not Affect FKBP12.6-RyR2 Interaction

It has been suggested that the phosphorylated serine-2808 of RyR2 may be located in close proximity to the binding pocket of FKBP12.6, such that static repulsion between the negatively charged phosphate group at serine-2808 of RyR2 and the negatively charged residue, aspartate-37, of FKBP12.6 causes FKBP12.6 to dissociate from RyR2. To examine the spatial and functional closeness between serine-2808 and FKBP12.6, we used a site-directed antibody that specifically recognizes the serine-2808 site. If serine-2808 is directly involved in FKBP12.6 binding, it would be expected that the binding of a large protein such as an antibody to the serine-2808 site would disrupt FKBP12.6 binding. To test this possibility, we purified antibodies against the S2808 site. The affinity-purified antibody interacted with both the phosphorylated and nonphosphorylated forms of serine-2808 peptide (Figures 6Aa and 6Ab) but did not recognize the S2808A mutant (Figure 6Ac), indicating that the antibody is specific in the intact RyR2 protein. The effect of this site-specific antibody on FKBP12.6-RyR2 interaction was examined in two ways. First, RyR2 was coexpressed with FKBP12.6 in HEK293 cells, and the resulting FKBP12.6-RyR2 complex was immunoprecipitated by an excess amount of the site-specific anti-serine-2808 antibody and then incubated with exogenous FKBP12.6. Figure 6Bb shows that the coexpressed FKBP12.6 remained bound to RyR2 after the addition of an excess amount of the anti-serine-2808 antibody (Figure 6Bb, lane 1) and that exogenous FKBP12.6 could still bind to the anti-serine-2808 antibody-bound RyR2 (Figure 6Bb, lane 2). These data indicate that the binding of an antibody to the serine-2808 site neither dissociates bound FKBP12.6 from RyR2 nor prevents FKBP12.6 from binding to RyR2, suggesting that the region encompassing serine-2808 is unlikely to be directly involved in FKBP12.6-RyR2 interaction.

In line with the above view, we have previously shown that the first 1855 NH2-terminal amino acid residues are sufficient for interaction with GST-FKBP12.6. Figures 6Ca and 6Cb show that this truncated RyR2, 1-1855, can also form a complex with FKBP12.6 both in vitro and in HEK293 cells.
FKBP12.6 was coimmunoprecipitated with the c-myc–tagged RyR2 (1-1855, c-myc) by the anti–c-myc antibody from HEK293 cells cotransfected with RyR2 (1-1855, c-myc) and FKBP12.6 (Figure 6Cb, lane 2). Exogenous FKBP12.6 can also interact with the expressed RyR2 (1-1855, c-myc) (Figure 6Cb, lane 4). On the other hand, a shorter truncated RyR2, 1-1815, did not form complexes with FKBP12.6. It should be noted that both the 1-1815–truncated and 1-1855–truncated RyR2 proteins, both of which lack the entire transmembrane domain, do not form a functional Ca$^{2+}$-release channel (not shown) and that both truncated RyR2 proteins lack the S2808 phosphorylation site. These results together with those reported previously suggest that the NH$_2$ terminal region, rather than the region encompassing the serine-2808, is critical for FKBP12.6 interaction.

FKBP12.6 Remains Bound to Native RyR2 Channels That Are Completely Phosphorylated by Exogenous PKA at Serine-2808

The results described above clearly demonstrate that PKA phosphorylation at serine-2808 does not dissociate recombinant FKBP12.6-RyR2 interaction. To investigate whether interaction between native RyR2 and FKBP12.6 can be disrupted by PKA phosphorylation, we isolated native FKBP12.6-RyR2 complex from canine heart microsomes by immunoprecipitation using the anti-RyR(34c) antibody. The immunoprecipitates were phosphorylated by exogenous PKA or by a boiled PKA and were separated into pellet and supernatant fractions by centrifugation. The pellet fractions of the immunoprecipitates treated with an active and inactive PKA were blotted with the anti-RyR(34c) (A), anti-S2808(PO$_3$) (B), or anti-S2808(deP) (C) antibody. The supernatant and pellet fractions of the immunoprecipitates treated with an active and inactive PKA were blotted with the anti-FKBP12.0/12.6 antibody (D).
the anti-RyR(34c) antibody. Interestingly, the native RyR2, with or without PKA phosphorylation, was recognized by the affinity-purified anti-S2808(PO3) antibody (Figure 7B, lanes 1 through 4), indicating that the native RyR2 was phosphorylated at serine-2808 before PKA treatment. The native RyR2 without PKA treatment or treated with an inactive PKA was recognized by the anti-S2808(deP) antibody (Figure 7C, lanes 1, 2, and 4), indicating that the native RyR2 was partially phosphorylated at serine-2808 under these conditions. On the other hand, the anti-S2808(deP) antibody did not react with the native RyR2 phosphorylated by exogenous FKBP12.6 (250 nmol/L), washed, and blotted with the anti-RyR(34c) antibody (Ba, lane 2) and the anti-FKBP12.0/12.6 antibody (Bb, lane 2). FKBP12.6 binding to truncated RyR2 is shown in C. The c-myc-tagged 1-1815 and 1-1855 RyR2 proteins coexpressed with FKBP12.6 were immunoprecipitated by the anti-c-myc antibody. The immunoprecipitates were blotted with the anti-c-myc antibody (Ca, lanes 1 and 2) or the anti-FKBP12.0/12.6 antibody (Cb, lanes 1 and 2). The c-myc-tagged 1-1815 and 1-1855 RyR2 proteins expressed alone were also immunoprecipitated by the anti-c-myc antibody. The immunoprecipitates were then mixed with exogenous FKBP12.6 (250 nmol/L), washed, and blotted with the anti-RyR(34c) (Ca, lanes 3 and 4) or the anti-FKBP12.0/12.6 (Cb, lanes 3 and 4) antibody.

FKBP12.6 was detected in the supernatant fractions of immunoprecipitates treated with an active or inactive form of PKA (Figure 7D, lanes 5 and 6), whereas strong FKBP12.6 signals were detected in the pellet fractions (Figure 7D, lanes 3 and 4). Figure 7D also shows that the native FKBP12.6-RyR2 interaction is sensitive to rapamycin (lane 2). These observations strongly indicate that a complete PKA phosphorylation of the native RyR2 at serine-2808 does not dissociate FKBP12.6.

Discussion
A fundamental aspect of the theory of PKA regulation of FKBP12.6-RyR2 interaction proposed by Marks et al to account for cardiac dysfunction in heart failure is that PKA phosphorylation of RyR2 at a single site, serine-2808 (or serine-2809 in rabbit RyR2), dissociates FKBP12.6 from RyR2. However, this key finding was questioned recently by...
Given the potential importance of this issue and the controversy surrounding it, we set out to additionally investigate the effect of PKA phosphorylation and mutations of RyR2 at serine-2808 on FKBP12.6-RyR2 interaction. Our data show that (1) FKBP12.6 is able to bind to both the serine-2808-phosphorylated and nonphosphorylated forms of RyR2; (2) FKBP12.6 can also bind to the S2808D mutant, which is thought to mimic constitutive phosphorylation; (3) complete phosphorylation at serine-2808 by exogenous PKA does not dissociate FKBP12.6 from either recombinant or native RyR2; and (4) binding of an antibody to the serine-2808 phosphorylation site does not prevent FKBP12.6 binding to or dissociate FKBP12.6 from RyR2. Taken together, the results of our present study do not support the notions that FKBP12.6 cannot bind to serine-2808-phosphorylated RyR2 or the S2808D mutant and that PKA phosphorylation of RyR2 at serine-2808 dissociates FKBP12.6.8,10

The reasons for the discrepancies between our studies and those of Marx et al8 and Wehrens et al10 are unclear. One potential factor that may account for these differences is the extent of phosphorylation of RyR2 by PKA. In the investigation of the relation between PKA phosphorylation and FKBP12.6 binding by Marx et al,8 Wehrens et al,10 and Jiang et al,12 the extent of PKA phosphorylation of RyR2, especially at serine-2808, was unclear. Different extents of PKA phosphorylation could potentially result in variable amounts of FKBP12.6 binding. To resolve this issue, we used a pair of antibodies that specifically recognize either the serine-2808 phosphorylated or serine-2808 nonphosphorylated form of RyR2 to unambiguously determine the phosphorylation state of serine-2808. Using these antibodies, we verified that the S2808A RyR2 mutant was completely nonphosphorylated, whereas RyR2 treated with exogenous PKA was completely phosphorylated at serine-2808. Hence, the inability of PKA phosphorylation to dissociate FKBP12.6 from RyR2 in our studies was not attributable to incomplete phosphorylation of RyR2 at serine-2808.

The source of the FKBP12.6-RyR2 complexes used for studying the impact of PKA phosphorylation may also have contributed to the controversy. In the study by Marx et al,8 PKA-induced FKBP12.6 dissociation was assessed using cardiac microsomal membranes, whereas detergent-solubilized and immunoprecipitated FKBP12.6-RyR2 complexes were used in the studies by Marx et al8 and Wehrens et al.10 It was suggested that the discrepancy could be related to detergent solubilization. However, the results of our present study show that this is not the case. PKA phosphorylation does not disrupt detergent-solubilized recombinant or native FKBP12.6-RyR2 complexes (Figures 5 and 7).

The form of FKBP12.6 and RyR2 proteins used could also be a concern. In our in vitro studies, we determined the interaction between a GST-FKBP12.6 fusion protein expressed in bacteria or FKBP12.6 derived from GST-FKBP12.6 after removal of GST by thrombin digestion and the mouse RyR2 expressed in HEK293 cells. These bacteria-expressed GST-FKBP12.6 or FKBP12.6 proteins may be different from the native FKBP12.6 with respect to binding to RyR2. In addition, the interaction between FKBP12.6-RyR2 is species-dependent.19 In the light of these concerns, we extended our studies using HEK293 cell–expressed FKBP12.6 and RyR2 and native canine FKBP12.6 and RyR2. Neither the recombinant FKBP12.6-RyR2 complex formed in HEK293 cells nor the native canine FKBP12.6-RyR2 complex was disrupted by PKA phosphorylation. Thus, the failure to detect PKA-induced FKBP12.6 dissociation in our studies did not result from the use of potentially abnormal forms of FKBP12.6 and RyR2 proteins.

It has recently been shown that a single point mutation at the serine-2808 phosphorylation site, S2808D, abolished FKBP12.6 binding, implying that residue serine-2808 is essential for FKBP12.6 interaction.15 This is surprising, given our previous observation that a large region in RyR2 (resi-
dues 1937 to 4967) encompassing the serine-2808 phosphorylation site and the previously mapped FKBP12.6 binding site (residues 2427 through 2428) is not required for FKBP12.6 binding to RyR2. To investigate the significance of the serine-2808 phosphorylation site in FKBP12.6 interaction, we have mutated serine-2808 of RyR2 to asparagine, S2808N. In contrast to the study by Wehrens et al., we found that both RyR2 wt and the S2808N mutant were able to interact with FKBP12.6. On the other hand, our results are consistent with those reported recently by Stange et al., who showed that the S2809D mutation in rabbit RyR2 did not abolish FKBP12.6 binding. The reasons for this discrepancy have yet to be determined. It has been suggested that the discrepancy may result from differences in experimental conditions, such as the solubilization condition. It was noted by Stange et al. that the solubilization conditions (0.25% Triton X-100 with low salt) used by Marx et al. were insufficient to solubilize RyRs from membranes.

Our results clearly indicate that FKBP12.6 can bind to serine-2808–phosphorylated RyR2 and the S2808D mutant and that PKA phosphorylation of RyR2 at serine-2808 does not dissociate FKBP12.6. However, our results cannot exclude the possibility that activation of the PKA pathway can dissociate FKBP12.6 from RyR2 in intact cardiac muscle or myocytes. In line with this view, it has recently been shown that treatment of HL-1 cardiac myocytes expressing RyR2 wt or mutants with isoproterenol or forskolin resulted in a dramatic reduction in the level of FKBP12.6 associated with the microsomal membranes, although the molecular events underlying the reduction of membrane-associated FKBP12.6 have yet to be determined. It is possible that dissociation of FKBP12.6 from RyR2 may require concomitant PKA phosphorylation of RyR2 and other proteins that may or may not associate with RyR2 or that activation of the PKA pathway may stimulate other signaling pathways, which, in turn, lead to FKBP12.6 dissociation. However, even if this is the case, our data at least indicate that PKA phosphorylation of RyR2 at serine-2808 in and of itself is insufficient to dissociate FKBP12.6 from RyR2.

In summary, using a pair of antibodies that recognize the serine-2808 phosphorylated and nonphosphorylated forms of RyR2, respectively, and mutants, which mimic these states, we were able to correlate for the first time the phosphorylation state of RyR2 at serine-2808 and its capability of binding FKBP12.6. We found no correlation between PKA phosphorylation of RyR2 at serine-2808 and FKBP12.6 binding. Additional investigations are needed to unveil the molecular mechanisms underlying the physiological regulation of FKBP12.6-RyR2 interaction and its roles in RyR2 and cardiac function.

Acknowledgments

This work was supported by research grants from the Canadian Institutes of Health Research to M.P.W. and S.R.W.C. B.X. is a recipient of the Alberta Heritage Foundation for Medical Research (AHFMR) Studentship Award. M.P.W. is an AHFMR Scientist and Canada Research Chair (Tier I) in Biochemistry. S.R.W.C. is an AHFMR Senior Scholar. We would like to thank Jeff Bolstad for critical reading of the manuscript and Cindy Brown for technical assistance.

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*Circ Res.* 2004;94:487-495; originally published online January 8, 2004;
doi: 10.1161/01.RES.0000115945.89741.22
*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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