Ser-2030, but not Ser-2808, is the major phosphorylation site in cardiac ryanodine receptors responding to protein kinase A activation upon β-adrenergic stimulation in normal and failing hearts

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We have recently shown that RyR2 (cardiac ryanodine receptor) is phosphorylated by PKA (protein kinase A/cAMP-dependent protein kinase) at two major sites, Ser-2030 and Ser-2808. In the present study, we examined the properties and physiological relevance of phosphorylation of these two sites. Using site- and phospho-specific antibodies, we demonstrated that Ser-2030 of both recombinant and native RyR2 from a number of species was phosphorylated by PKA, indicating that Ser-2030 is a highly conserved PKA site. Furthermore, we found that the phosphorylation of Ser-2030 responded to isoproterenol (isoprenaline) stimulation in rat cardiac myocytes in a concentration- and time-dependent manner, whereas Ser-2808 was already substantially phosphorylated before β-adrenergic stimulation, and the extent of the increase in Ser-2030 phosphorylation after β-adrenergic stimulation was much less than that for Ser-2030. Interestingly, the isoproterenol-induced phosphorylation of Ser-2030, but not of Ser-2808, was markedly inhibited by PKI, a specific inhibitor of PKA. The basal phosphorylation of Ser-2808 was also insensitive to PKA inhibition. Moreover, Ser-2808, but not Ser-2030, was stoichiometrically phosphorylated by PKG (protein kinase G). In addition, we found no significant phosphorylation of RyR2 at the Ser-2030 PKA site in failing rat hearts. Importantly, isoproterenol stimulation markedly increased the phosphorylation of Ser-2030, but not of Ser-2808, in failing rat hearts. Taken together, these observations indicate that Ser-2030, but not Ser-2808, is the major PKA phosphorylation site in RyR2 responding to PKA activation upon β-adrenergic stimulation in both normal and failing hearts, and that RyR2 is not hyperphosphorylated by PKA in heart failure. Our results also suggest that phosphorylation of RyR2 at Ser-2030 may be an important event associated with altered Ca2+ handling and cardiac arrhythmia that is commonly observed in heart failure upon β-adrenergic stimulation.

Key words: heart failure (HF), β-adrenergic stimulation, protein kinase A (PKA), ryanodine receptor (RyR) phosphorylation, phospho-specific antibodies.

INTRODUCTION

PKA (protein kinase A/cAMP-dependent protein kinase) is a key regulator of Ca2+ handling in the heart. Activation of PKA as a result of β-adrenergic receptor stimulation and the consequent increase in the level of cAMP leads to the phosphorylation of several major Ca2+ cycling proteins in cardiac cells, including the L-type Ca2+ channel, phospholamban, and RyR2 (cardiac ryanodine receptor) [1]. Phosphorylation of the L-type Ca2+ channel by PKA increases the functional availability and open-probability of the channel [2,3], leading to enhanced Ca2+ influx, whereas phosphorylation of phospholamban by PKA relieves its tonic inhibition of the SR (sarcoplasmic reticulum) Ca2+-ATPase, thus increasing Ca2+ uptake into the SR [4]. The net result is an increase in the SR Ca2+ load, SR Ca2+ release, and cardiac output. The molecular basis of phosphorylation and regulation of RyR2 by PKA, however, remains undefined and highly controversial [5].

Marks et al. [6,7] reported that RyR2 is phosphorylated by PKA at a single residue, Ser-2808 (Ser-2809 in rabbit RyR2), which was originally identified as a unique CaMKII (Ca2+- and calmodulin-dependent protein kinase II) phosphorylation site [8,9]. They showed that a single point mutation of Ser-2808 in a fragment of RyR2, when expressed in the form of a GST (glutathione-S-transferase)-fusion protein, abolished PKA phosphorylation, and that the stoichiometry of phosphorylation of the human RyR2 by PKA was approx. 1 phosphate per RyR2 monomer [6]. Recently, they reported that a single point mutation, S2808A, in the full-length recombinant human RyR2 abolished phosphorylation of RyR2 by PKA [7]. Moreover, RyR2 proteins isolated from knock-in mice harbouring the S2808A mutation were no longer phosphorylated by PKA [10,11]. Based on these observations, they proposed that Ser-2808 is the sole PKA phosphorylation site in RyR2, and that it mediates the action of PKA in RyR2 channel regulation.

In contrast with these findings, tryptic phosphopeptide mapping of canine RyR2, which is phosphorylated by PKA, revealed one major and two minor phosphorylated peptides, suggesting the existence of multiple PKA phosphorylation sites in native canine RyR2 [12]. In support of this notion, we have recently identified a novel PKA phosphorylation site, Ser-2030, in the recombinant mouse RyR2 in addition to the previously identified phosphorylation site, Ser-2808 [13]. Using a phospho-specific antibody against Ser-2030, we further demonstrated that RyR2 was phosphorylated at Ser-2030 in rat cardiac myocytes and in canine hearts treated with isoproterenol (isoprenaline), indicating that the Ser-2030 PKA phosphorylation site is physiologically relevant [13]. The reason for this discrepancy regarding the number of phosphorylation sites in RyR2 needs to be clarified.
PKA phosphorylation sites in RyR2 is unclear, and further investigations are needed to resolve this issue.

The phosphorylation status of RyR2 by PKA in normal and diseased hearts is also controversial. It has been shown that RyR2 is hyperphosphorylated by PKA in failing human, canine and rat hearts [6,7,14]. This is thought to be the result of an elevated level of circulating catecholamines that occurs in HF (heart failure). This hyperphosphorylation of RyR2 by PKA has been proposed as a major mechanism underlying cardiac dysfunction in HF. Since Ser-2808 was believed to be the only PKA site in RyR2, the phosphorylation status of Ser-2808 has been widely used as an index for RyR2 phosphorylation by PKA. However, Jiang et al. [15] demonstrated that there are no measurable differences in the phosphorylation level of RyR2 by PKA between failing and non-failing canine hearts. Using phospho-specific antibodies against Ser-2030 and Ser-2808, we have recently shown that there is no hyperphosphorylation of RyR2 by PKA in canine HF [13]. In our previous studies, the phosphorylation status of RyR2 was determined using whole-heart homogenates, whereas immunoprecipitated RyR2 proteins from detergent-solubilized SR membranes or heart tissue homogenates were used for measuring phosphorylation levels in the studies by Marks et al. [6,7]. Hence, it is possible that differences in experimental conditions may contribute, in part, to the controversy regarding the phosphorylation status of RyR2 by PKA in HF. Another potential source of this discrepancy is the use of Ser-2808 phosphorylation as an index for RyR2 phosphorylation by PKA. It is known that Ser-2808 is phosphorylated by both PKA and CaMKII, and that Ser-2808 is substantially phosphorylated at rest, in the absence of β-adrenergic stimulation, indicating that the phosphorylation of Ser-2808 is regulated not only by PKA but also by other kinases [13,16,17]. It is therefore unclear whether Ser-2808 phosphorylation truly reflects the phosphorylation status of RyR2 by PKA, and whether Ser-2808 mediates the action of PKA in modulating RyR2 channel activity.

In light of these questions and controversial issues, it is imperative to understand the molecular basis of RyR2 phosphorylation and regulation by PKA both in vitro and in vivo. In the present study, we have investigated whether RyR2s from various species can be phosphorylated at the newly identified site, Ser-2030. We have determined the response of Ser-2030 and Ser-2808 phosphorylation to β-adrenergic stimulation in cardiac myocytes and the effect of various kinase inhibitors on Ser-2030 and Ser-2808 phosphorylation. We have also assessed the phosphorylation status of Ser-2030 and Ser-2808 in normal and failing hearts with or without β-adrenergic stimulation. Our results demonstrate that Ser-2030 is a highly conserved PKA phosphorylation site in RyR2, and that the phosphorylation of Ser-2030, but not of Ser-2808, closely correlates with β-adrenergic stimulation in cardiac myocytes and in normal and diseased hearts. In addition, we found no significant phosphorylation of RyR2 by PKA in HF. Our data suggest that Ser-2030 is the major RyR2 PKA phosphorylation site that is linked to β-adrenergic stimulation and PKA activation in normal and failing hearts, in contrast with the common belief that Ser-2808 is the only RyR2 phosphorylation site that mediates the action of PKA.

**EXPERIMENTAL**

**Materials**

An anti-RyR antibody (34C), which recognizes all three isoforms of RyR, was purchased from Affinity Bioreagents Inc. (Golden, CO, U.S.A.). An anti-RyR2 antibody, which specifically recognizes the RyR2 isoform, was kindly provided by Dr F. Anthony Lai (Department of Cardiology, Cardiff University, U.K.). An anti-Ser-2808 (dPO3) antibody, which specifically recognizes RyR2 unphosphorylated at Ser-2808, was purchased from Badrilla (U.K.). The generation and characterization of anti-Ser-2030 (PO4) and anti-Ser-2808 (PO4) antibodies, which specifically recognize RyR2 phosphorylated at Ser-2030 and Ser-2808, respectively, have been described previously [13,18]. PKA was purified from bovine hearts according to the method of Demaille et al. [19]. PKG (protein kinase G) and calyculin A were obtained from Upstate. PKI-(14-22) (myristoylated) (where PKI is a specific inhibitor of PKA) and KN93 were from Calbiochem. CHAPS, H89, KT5823, staurosporine and other reagents were purchased from Sigma. Plant phosphatidylcholine was from Avanti Polar Lipids.

**DNA transfection**

Full-length mouse RyR2 cDNA was cloned and constructed as described previously [20,21]. Full-length rabbit and human RyR2 cDNA clones were kindly provided by Dr David H. MacLennan (Banting and Best Department of Medical Research, University of Toronto, Canada) and Dr F. Anthony Lai (Cardiff University) respectively. HEK (human embryonic kidney) 293 cells grown on 100 mm tissue culture dishes in supplemented DMEM (Dulbecco’s modified Eagle’s medium) for 18–20 h after subculture were transfected with RyR2 cDNA or co-transfected with RyR2 cDNA (12 µg) and cDNA encoding the catalytic subunit of PKA (4 µg) using the calcium phosphate precipitation method as described previously [21].

**Preparation of cell lysates from transfected HEK293 cells and isolated cardiac myocytes**

HEK293 cells grown for 24–26 h after transfection were washed three times with PBS (137 mM NaCl, 8 mM Na2HPO4, 1.5 mM KH2PO4 and 2.7 mM KCl) plus 2.5 mM EDTA, and were harvested in the same solution by centrifugation for 8 min at 700 g in an IEC Centra-CL2 (International Equipment Co., Needham Heights, MA, U.S.A.) centrifuge. Cell pellets were solubilized in a lysis buffer containing 25 mM Tris/50 mM Hepes (pH 7.4), 137 mM NaCl, 1% CHAPS, 0.5% soybean phosphatidylcholine, 2.5 mM DTT (dithiothreitol) and a protease inhibitor mix (1 mM benzamidine, 2 µg/ml leupeptin, 2 µg/ml pepstatin A, 2 µg/ml aprotinin and 0.5 mM PMSF). Rat cardiac myocytes were pelleted by centrifugation at 16000 g for 30 s. Cell pellets were solubilized in a solubilization buffer containing 50 mM Tris/HCl (pH 7.4) and 2% SDS. This mixture was incubated on ice for 1 h. Cell lysates from both HEK293 cells and cardiac myocytes were obtained by centrifugation at 16000 g in a microcentrifuge at 4 °C for 30 min performed twice to remove the unsolubilized materials.

**Treatment of cardiac myocytes and HEK293 cells**

Mouse cardiac myocytes were isolated and cultured as described previously [22]. In some experiments, cultured mouse cardiac myocytes were treated with 1 µM isoproterenol for different periods of time. In other experiments, cultured myocytes were first pre-treated with 10 µM PKI for 30 min, then 1 µM isoproterenol was added and cells were incubated for different periods of time in the continued presence of PKI. Myocytes were harvested, quickly frozen in liquid nitrogen and stored at −80°C until further use. To investigate the concentration-dependence and time-course of phosphorylation upon isoproterenol stimulation, aliquots of freshly isolated rat cardiac myocytes were treated with various concentrations (0 to 1 µM) of isoproterenol for 15 min, or with 100 nM isoproterenol for different periods of time (0 to 60 min). To study the effect of inhibition of protein phosphatases on RyR2...
phosphorylation, freshly isolated rat cardiac myocytes were pre-
incubated with or without 20 nM calyculin A for 30 min, then
stimulated with or without 100 nM isoproterenol for 15 min.
Myocytes were then centrifuged at 15 000 g for 30 s. Cell pellets
were lysed in a buffer containing 50 mM Tris/HCl (pH 7.4) and
2 % SDS. The resulting cell lysates were used for immunoblotting.
To study the effect of protein kinase inhibitors on the basal
phosphorylation level of Ser-2808, HEK293 cells were grown in
12-well plates and transfected with 12 µg of mouse RyR2 cDNA.
At 24 h after transfection, KN93 (10 µM), H89 (1 µM), KT823
(50 µM) or staurosporine (1 µM) was added to the cell culture
and incubated at 37 °C for 30–60 min. Cells were then lysed with
100 µl of boiled 1 × Laemmli’s sample buffer. The resulting cell
lysates were used for immunoblotting studies. Similar results were
obtained with cells to which the protein kinase inhibitors were added 1 h after transfection and incubated at 37 °C for 24 h.

Preparation of cardiac muscle homogenates
All studies with rats were approved by the Animal Care Com-
mitee of the University of Calgary and complied with the Guide
for the Care and Use of Laboratory Animals published by the US
National Institutes of Health. Human left ventricular tissues from
non-failing donor hearts were obtained with informed consent and
approved by the Institutional Review Board (provided by Dr Ming
Tao Jiang, Medical College of Wisconsin, U.S.A.). The protocols
for induction and characterization of the rat HF model with CMI
(congestive myocardial infarction) have been described in detail
elsewhere [23]. To preserve the native phosphorylation status of
RyR2, tissues blocks were crushed by a Wollenberger clamp
pre-cooled in liquid nitrogen and stored at −80 °C until further
use. Frozen cardiac tissues were pulverized in liquid nitrogen and
homogenized immediately with a Brinkmann Polytron PT
15 homogenizer (setting 8, 4 bursts for 15 s each) in 6 vol. of
30 mM KH2PO4 (pH 7.0), 40 mM NaF, 5 mM EDTA, 300 mM
sucrose, 4 µM leupeptin, 1 mM benzamidine, 100 µM PMSF
and 0.5 mM DTT. Aliquots of homogenates were solubilized in
50 mM Tris/HCl (pH 7.4) plus 3 % SDS for 1 h at room tem-
perature. The insoluble materials were then removed by centrifu-
gation at 4000 g for 10 min. The protein concentration of the
supernatant was determined using a Bio-Rad detergent-com-
patible protein assay kit. Aliquots (20–30 µg) of the protein were
used for SDS/PAGE and immunoblotting.

Protein kinase assays
RyR2 proteins immunoprecipitated with an anti-RyR antibody
were subjected to phosphorylation by PKA or PKG. Phosphoryl-
ation of RyR2 by PKA was carried out in a buffer containing
(in mM): 25 Tris/50 Hepes (pH 7.4), 1 EGTA, 6 Mg2+, 10 NaF
and 1 µg/ml of the PKA catalytic subunit with or without 5 µM
PKI, or with the same amount of the PKA catalytic subunit
which had been boiled for 30 min. Phosphorylation of RyR2 by
PKG was conducted in a buffer containing (in mM): 40 Mops
(pH 7.4), 1 EDTA, 50 NaF, 10 Mg2+, 0.01 cGMP, and 0.4 µg/ml
of active PKG or the same amount of PKG which had been boiled
for 30 min. Phosphorylation reactions were initiated by adding
0.5 mM ATP and were carried out at 30 °C for 30 min. After
phosphorylation, the beads were centrifuged and solubilized in an
equal volume of 2 × Laemmli’s SDS sample buffer. The solubi-
lized samples were subjected to SDS/PAGE and immunoblotting
as previously described [18].

Immunoprecipitation of RyR2
Immunoprecipitation of RyR2 was carried out as described
previously [18]. Briefly, HEK293 cell lysates or solubilized heart
homogenates were incubated with Protein G–Sepharose (15 µl)
pre-bound with 2 µg of the anti-RyR antibody at 4 °C for 17–19 h. The Protein G-immunoprecipitates were washed with ice-cold
lysis buffer containing the protease inhibitor mix (as described
above) for 10 min, three times. Proteins bound to the Sepharose
beads were solubilized in 20 µl of 2 × Laemmli’s sample buffer
[24] plus 5 % 2-mercaptoethanol and boiled for 5 min. An equal
portion of the solubilized proteins from different samples was
then subjected to SDS/(6 %) PAGE.

Immunoblotting analyses
SDS/PAGE resolved proteins were transferred to nitrocellulose
membranes at 45 V for 18–20 h at 4 °C in the presence of 0.01 %
SDS according to the method of Towbin et al. [25]. The nitro-
cellulose membranes containing the transferred proteins were
blocked for 30 min with PBS containing 0.5 % Tween-20 and 5 %
skimmed-milk powder. The blocked membrane was incubated
with anti-RyR2 (1:1000), anti-Ser-2030(PO3) (1:1000), anti-Ser-
2808(PO3) (1:5000), or anti-Ser-2808(PO3) (1:5000) antibodies,
and washed with PBS containing 0.5 % Tween-20 for 15 min,
three times. The membrane was then incubated with the secondary
anti-mouse or anti-rabbit IgG (H&L) antibodies conjugated to
horseradish peroxidase (1:20 000), for 30 min. After washing for
15 min, three times, the bound antibodies were detected using an
enhanced chemiluminescence kit from Pierce.

Quantification of the phosphorylation level of RyR2
at Ser-2808 and Ser-2030
SDS/PAGE gels (3 sets) with identical loadings were immunoblot-
ted with anti-RyR2, anti-Ser-2030(PO3) and anti-Ser-2808(PO3)
antibodies respectively. Aliquots (20–30 µg) of proteins from
SDS-solubilized cardiac myocytes or left ventricle homogenates
were loaded in each lane. In some experiments, the same immuno-
blot was probed and re-probed with the anti-Ser-2030(PO3), anti-
RyR2 and anti-Ser-2808(PO3) antibodies sequentially after stripp-
ing off the previously bound antibodies. Similar results were
obtained using both methods. The immunoblotting results shown
in Figures 3, 4, 6, 7, 8 and 9 were obtained using the latter
method. The relative levels of phosphorylation were determined
by densitometric analyses of the antibody signals using a Bio-
Rad Scanner and the Imagemaster program, and expressed as
ratio of intensity of the anti-Ser-2808(PO3) antibody or the anti-
Ser-2030(PO3) antibody signal to the anti-RyR2 antibody signal.
Data points shown are means ± S.E.M. Statistical significance
was evaluated using the unpaired Student’s t test. Differences
were considered significant when P < 0.05.

Stripping of immunoblot membranes
Following immunoblot analysis with one antibody, membranes
were incubated at 55 °C in a stripping buffer containing 50 mM
Tris/HCl (pH 6.8), 2 % SDS and 0.1 M 2-mercaptoethanol for
30 min. Membranes were then washed with PBS for 5 min, three
times, and re-probed with another antibody.

RESULTS
Native mouse RyR2 is phosphorylated by PKA at both Ser-2030
and Ser-2808 residues
We have recently identified a novel PKA phosphorylation site,
Ser-2030, in recombinant mouse RyR2 expressed in HEK293
cells [13]. More recently, however, Lehnart et al. [10,11] re-
ported that RyR2 isolated from knock-in mice containing a
RyR2 phosphorylation site mutation, S2808A, was no longer
phosphorylated by PKA in vitro, suggesting that Ser-2030 is the only PKA phosphorylation site in native mouse RyR2. This observation is inconsistent with our recent finding that recombiant mouse RyR2 is phosphorylated by PKA at two major sites, Ser-2030 and Ser-2808. To determine whether or not native mouse RyR2 is phosphorylated by PKA at Ser-2030, we phosphorylated RyR2, immunoprecipitated from a mouse heart homogenate, by PKA in vitro. We detected the phosphorylation of Ser-2030 in mouse RyR2 treated with the catalytic subunit of PKA (Figure 1B, lane 1), but not in mouse RyR2 treated with boiled (inactive) PKA (Figure 1B, lane 2). On the other hand, phosphorylation of mouse RyR2 at Ser-2808 was readily detected in the presence of inactivated PKA and was increased after treatment with active PKA (Figure 1C, lanes 1 and 2 respectively). These data clearly indicate that native mouse RyR2, like recombinant mouse-RyR2, is phosphorylated in vitro by PKA at both Ser-2030 and Ser-2808.

**Ser-2030 is a conserved PKA phosphorylation site in RyR2s from various species**

To investigate whether RyR2s from other species are also phosphorylated by PKA at Ser-2030, we expressed rabbit and human RyR2 in HEK293 cells and immunoprecipitated the expressed RyR2. The immunoprecipitated RyR2 was phosphorylated by PKA in vitro in the presence or absence of PKI-(1-42), a specific PKA inhibitor, or by inactive PKA (boiled for 30 min). As shown in Figure 2A(b), rabbit and human RyR2 expressed in HEK293 cells, like the expressed mouse RyR2, was phosphorylated by PKA at Ser-2030. Phosphorylation of Ser-2030 was not detected in the presence of PKI or boiled PKA, indicating that the phosphorylation of Ser-2030 is dependent on the activity of PKA and is not due to the action of other kinases that might have been co-immunoprecipitated with RyR2 (Figure 2A, b). As in the case of mouse RyR2, PKA treatment also increased the level of phosphorylation of rabbit and human RyR2s at Ser-2808, but there was a considerable basal level of phosphorylation of rabbit and human RyR2s at Ser-2808 before PKA treatment (Figure 2A, c).

We next determined whether Ser-2030 in rabbit and human RyR2 could be phosphorylated by PKA in a cellular environment. HEK293 cells were co-transfected with mouse, rabbit or human RyR2 cDNA and the cDNA encoding the PKA catalytic subunit. The expressed RyR2 proteins were immunoprecipitated and the phosphorylation status of Ser-2030 and Ser-2808 was assessed. As seen in Figure 2B, the phosphorylation of rabbit and human RyR2 at Ser-2030 was only detected when RyR2 was co-expressed with PKA (Figure 2B, lane 2). There was no detectable phosphorylation at Ser-2030 when RyR2 was expressed alone (Figure 2B, lane 1). The results demonstrate that like mouse RyR2, rabbit and human RyR2 is also phosphorylated by PKA at Ser-2030 in HEK293 cells. Unlike Ser-2030, for which phosphorylation is dependent upon the co-expression of PKA, Ser-2808 was phosphorylated with or without co-expression of PKA (Figure 2B, c) in HEK293 cells.

Like recombinant RyR2, native rabbit and human RyR2 are also phosphorylated by PKA at both Ser-2030 and Ser-2808. Figure 2C shows that RyR2 immunoprecipitated from rabbit and human heart homogenates was phosphorylated by PKA at Ser-2030 (Figure 2C, b). Similarly, a considerable basal level of phosphorylation of Ser-2808 was also observed in native rabbit and human RyR2 before PKA treatment (Figure 2C, c). Taken together, these data demonstrate that Ser-2030 is a conserved PKA site in RyR2. These data also show that Ser-2808 of RyR2 from various species is substantially phosphorylated at rest in both a heterologous expression system and in native tissues, suggesting that Ser-2808 of RyR2 is constitutively phosphorylated.
Phosphorylation of RyR2 by PKA in non-failing and failing hearts

Figure 3 Phosphorylation of Ser-2030 and Ser-2808 in response to isoproterenol stimulation in rat cardiac myocytes

Freshly isolated rat cardiac myocytes were stimulated with various concentrations of isoproterenol (Iso; 0–1 μM) for 15 min (A) or with 100 nM isoproterenol for different periods of time (0–60 min) (B). Whole-cell lysates were prepared with 2% SDS and immunoblotted using anti-RyR2 (a), anti-Ser-2030(PO3) (b), or anti-Ser-2808(PO3) (c) antibodies. The relative phosphorylation level was assessed by determining the ratio of the anti-Ser-2030(PO3) signal or the anti-Ser-2808(PO3) signal to the anti-RyR2 signal. (B, d) (100%). (d, upper and lower) show summary data from four separate experiments. (*P < 0.05 versus isoproterenol treatment at 0 nM or at 0 min).

Differential phosphorylation of Ser-2030 and Ser-2808 in cardiac myocytes in response to isoproterenol stimulation

The observation that Ser-2808, but not Ser-2030, is substantially phosphorylated at rest suggests that the phosphorylation of Ser-2030 and Ser-2808 may be differentially regulated. To test this hypothesis, we assessed the phosphorylation status of Ser-2030 and Ser-2808 in freshly isolated rat cardiac myocytes stimulated with isoproterenol. Figure 3(A) shows the concentration-dependence of the phosphorylation of Ser-2030 and Ser-2808 in response to isoproterenol stimulation. The phosphorylation of Ser-2030 was barely detectable in the absence of isoproterenol stimulation, but treatment with increasing concentrations of isoproterenol from 10 nM to 1 μM dramatically increased Ser-2030 phosphorylation in a concentration-dependent manner (Figure 3A, panels b and d). On the other hand, Ser-2808 was already substantially phosphorylated before isoproterenol stimulation, and the extent of the increase in Ser-2808 phosphorylation with increasing concentrations of isoproterenol was much less than that for Ser-2030 (Figure 3A, panels c and d). Figure 3(B) shows the time course of phosphorylation of Ser-2030 and Ser-2808 in response to isoproterenol stimulation. As occurred with the concentration dependence, the phosphorylation of Ser-2030 increased progressively and markedly with increasing stimulation time (0–60 min) (Figure 3B, panels b and d), whereas the increase in Ser-2808 phosphorylation was relatively small (Figures 3B, c and 3B, d). These observations demonstrate that Ser-2030 differs from Ser-2808 with respect to the concentration dependence and time-course of isoproterenol-induced phosphorylation, and that the phosphorylation of RyR2 at Ser-2030 is more closely correlated with β-adrenergic stimulation than is phosphorylation at Ser-2808.

Isoproterenol-induced phosphorylation of Ser-2030, but not of Ser-2808, is markedly inhibited by PKI

The unique phosphorylation patterns of Ser-2030 and Ser-2808 in response to isoproterenol stimulation suggest that Ser-2030 and Ser-2808 phosphorylation may be regulated by different kinases. To address this possibility, we pre-treated mouse cardiac myocytes with or without 10 μM PKI for 30 min, followed by isoproterenol (1 μM) stimulation for various periods of time. As seen in Figure 4, the level of the RyR2 protein in all these conditions was comparable (Figure 4A). Isoproterenol stimulation for 10 min or 1 h markedly increased the phosphorylation level of RyR2 at Ser-2030 (Figure 4B, lanes 2 and 3). However, prolonged stimulation with isoproterenol for 8 h led to a decrease in Ser-2030 phosphorylation compared with that observed after 10 min of isoproterenol stimulation (to 59.4 ± 12.9%, n = 3) (P < 0.05) (Figure 4B, lane 4). This is likely to be the result of down-regulation and/or desensitization of the β-adrenergic receptor/AMP/PKA signalling pathway during sustained β-adrenergic stimulation. Importantly, the isoproterenol-induced phosphorylation of Ser-2030 was markedly inhibited by PKI. The extent of phosphorylation at Ser-2030 after 10 min or 1 h of isoproterenol stimulation in the presence of PKI was 29.5 ± 6.8% (n = 3, P < 0.001) and 60.3 ± 3.1% (n = 3, P < 0.05) of that in the absence of PKI respectively (Figure 4B, lanes 6 and 7). By contrast, both the basal and isoproterenol-stimulated phosphorylation of Ser-2808 was not significantly inhibited by PKI (Figure 4C, lanes 5–7). Moreover, unlike Ser-2030 phosphorylation, prolonged isoproterenol stimulation did not lead to a significant decrease in Ser-2808 phosphorylation.
In lane 1, mouse RyR2 expressed in HEK293 cells was immunoprecipitated with an anti-RyR2 antibody. Aliquots of immunoprecipitated RyR2 were treated with PKG (lane 2), boiled PKG (lane 3), PKA (lane 4) or with no treatment (lane 1). The samples were then immunoblotted with anti-RyR2 (A, anti-Ser-2030(PO3)), (A, c), or anti-Ser-2030(PO3)(A_d) antibodies. Note that Ser-2030, but not Ser-2030, was completely phosphorylated by PKG, judging from the absence of an anti-Ser-2030(PO3) signal. In (B), HEK293 cells transfected with mouse RyR2 were treated with KN93 (lane 2), H89 (lane 3), KT5823 (lane 4) or staurosporine (STS) (lane 5) for 30 min. Whole-cell lysates were prepared and immunoblotted using anti-RyR2 (B, a) or anti-Ser-2030(PO3) (B, b) antibodies. Similar results were obtained from three separate experiments.

Ser-2030 is a substrate for multiple protein kinases
Ser-2030 is a consensus phosphorylation site (RRISer-2030) not only for PKA (RRXS/T), but also for CaMKII (RXXS/T) and PKG (RR/KXS/T) [26]. Indeed, it has been shown that CaMKII is able to phosphorylate Ser-2030, but not Ser-2030 [8,13,17]. To examine whether Ser-2030 can also be phosphorylated by PKG, we expressed RyR2 in HEK293 cells and immunoprecipitated the expressed RyR2. As shown in Figure 5, a comparable amount of RyR2 protein was used for each condition (Figure 5A, a). Treatment with PKG led to a marked increase in Ser-2030 phosphorylation (Figure 5A, b; lane 2), and the disappearance of non-phosphorylated Ser-2030, as revealed by the anti-Ser-2030(dPO3) antibody (Figure 5A, c; lane 2), which specifically recognizes the non-phosphorylated Ser-2030. These observations indicate that PKG, like PKA (Figure 5A, b and c; lane 4), is able to completely phosphorylate RyR2 at Ser-2030. It should be noted that no increase in Ser-2030 phosphorylation was detected when treated with inactive (boiled) PKG (Figure 5A, b and c; lane 3). On the other hand, PKG phosphorylated RyR2 at Ser-2030 only weakly compared with PKA (Figure 5A, d; lanes 2 and 4). Thus multiple kinases including PKA, CaMKII and PKG are able to phosphorylate RyR2 at Ser-2030.

Effect of various protein kinase inhibitors on the basal phosphorylation of Ser-2030
In an attempt to identify the kinase(s) responsible for the basal phosphorylation of Ser-2030 at rest, we transfected HEK293 cells with RyR2 cDNA in the presence or absence of KN93 (a CaMKII inhibitor), H89 (a PKA inhibitor), KT5823 (a PKG inhibitor) or staurosporine (a non-specific protein kinase inhibitor). Figure 5(B) shows that KN93 (Figure 5B, b; lane 2), H89 (Figure 5B, b; lane 3), and KT5823 (Figure 5B, b; lane 4) did not suppress the basal phosphorylation level of RyR2 at Ser-2030, whereas staurosporine markedly decreased the basal phosphorylation level of Ser-2030 (Figure 5B, b; lane 5). These data suggest that CaMKII, PKA and PKG, although they are able to phosphorylate Ser-2030, are not the major kinases responsible for the basal phosphorylation of Ser-2030. The major kinase(s) that phosphorylates Ser-2030 at rest and during β-adrenergic stimulation has yet to be identified.

Effect of inhibition of phosphatases on the phosphorylation of Ser-2030 and Ser-2030
To determine whether the phosphorylation of Ser-2030 and Ser-2030 is differentially regulated not only by kinases, but also by protein phosphatases, we assessed the effect of calyculin A, a membrane-permeable inhibitor of the protein phosphatases, PP1 and PP2A, both of which have been shown to physically interact with RyR2 [6], on the phosphorylation level of Ser-2030 and Ser-2030. As seen in Figure 6, treatment with calyculin A (20 nM) increased the basal phosphorylation level of Ser-2030 (to 147.8 ± 9.7 %, n = 5, P < 0.005) (Figure 6C, lane 2), but did not affect that of Ser-2030 (Figure 6B, lane 2). On the other hand, calyculin A did not appear to significatly affect the isoproterenol (100 nM)-induced phosphorylation of either Ser-2030 (100 % versus 79.8 ± 19.3 %, n = 4, P = 0.34) (Figure 6B, lanes 3 and 4) or Ser-2030 (166.3 ± 29.0 % versus 149.3 ± 11.5 %, n = 5, P = 0.57) (Figure 6C, lanes 3 and 4) (Figures 6D and 6E). Interestingly, the increase in basal phosphorylation of Ser-2030 as a result of calyculin A treatment is similar to that induced by isoproterenol (147.8 ± 9.7 % versus 166.3 ± 29.0 %, n = 5, P = 0.53). These data suggest that the basal phosphorylation of...
Ser-2808 is subject to regulation by both protein kinase(s) and phosphatase(s).

**RyR2 is not hyperphosphorylated by PKA in failing rat hearts**

The phosphorylation status of RyR2 by PKA in a rat model of HF was examined using an anti-Ser-2030(PO3) antibody. As seen in Figure 7, although the RyR2 protein was clearly detected by the anti-RyR2 antibody (Figure 7A), very weak phosphorylation of RyR2 at Ser-2030 was observed in both failing and non-failing rat hearts (Figure 7C), indicating that there is no hyperphosphorylation of RyR2 by PKA in failing rat hearts. On the other hand, RyR2 was considerably phosphorylated at Ser-2030 in both failing and non-failing rat hearts (Figure 7B). Analysis of the ratios of these phosphorylation signals revealed that the phosphorylation of Ser-2030 in failing rat hearts was significantly increased compared with that in non-failing rat hearts (157.8 ± 5.6 %, n = 4) (P < 0.005) (Figure 7D). However, considering the observations that there is very little phosphorylation at the PKA-specific site, Ser-2030, and that Ser-2030 can be phosphorylated by multiple kinases and its phosphorylation is tightly regulated by phosphatases, the increase in Ser-2030 phosphorylation is unlikely to be attributable to PKA. Together with the results of our previous studies on canine HF [13], these data clearly demonstrate that RyR2 is not hyperphosphorylated by PKA in HF.

**Ser-2030 and Ser-2808 are phosphorylated in rat hearts in vivo in response to isoproterenol stimulation**

The lack of phosphorylation at Ser-2030 in non-failing and failing rat heart samples raises the concern that the anti-Ser-2030(PO3) antibody used may not be sensitive enough to detect the phosphorylation of Ser-2030 in heart tissues. To address this concern, we injected normal rats with isoproterenol (10 µg/kg body weight) for 15 min. Their hearts were then quickly removed and flash-frozen in liquid nitrogen. As shown in Figure 8(B), control rat hearts without injection of isoproterenol showed weak phosphorylation of RyR2 at Ser-2030 (Figure 8B, lanes 1–3), whereas isoproterenol-injected rat hearts displayed strong phosphorylation of Ser-2030 (Figure 8B, lanes 4–6). These data confirm that the anti-Ser-2030 antibody is able to detect Ser-2030 phosphorylation in heart tissues. The phosphorylation status of Ser-2030 in intact rat hearts injected with or without isoproterenol is shown in Figure 8(C). The phosphorylation of Ser-2030 was readily detected in rat hearts without isoproterenol treatment, and was significantly increased (to 170 ± 13.9 %, n = 3) (P < 0.05) after isoproterenol treatment. These results demonstrate that both Ser-2030 and Ser-2808 are phosphorylated in rat hearts in vivo in response to β-adrenergic receptor activation.

**Isoproterenol treatment markedly increases the phosphorylation of Ser-2030, but not of Ser-2808, in failing rat hearts in vivo**

The phosphorylation of RyR2 at Ser-2030 and Ser-2808 in response to β-adrenergic stimulation was also examined in rats with HF. As with normal rats, the phosphorylation of Ser-2030 was weakly detected in failing rat hearts without isoproterenol treatment, and was markedly increased after isoproterenol injection (10 µg/kg body weight) (Figure 9B). On the other hand, isoproterenol treatment slightly increased the phosphorylation of Ser-2808 in failing rat hearts (112.7 ± 2.9 %, n = 3) (P < 0.05) (Figures 9C and 9D). These observations indicate that the phosphorylation of RyR2 at Ser-2030 in failing rat hearts still responds strongly to β-adrenergic stimulation. On the other hand, the phosphorylation of Ser-2808 in failing rat hearts responded weakly to β-adrenergic stimulation.

**DISCUSSION**

It is widely believed that Ser-2808 is the only PKA phosphorylation site in RyR2, and that the phosphorylation of RyR2 at Ser-2808 is an essential step in the β-adrenergic receptor/PKA
signalling pathway that regulates RyR2 channel activity and thus SR Ca\(^{2+}\) release and cardiac contractility [7,10,11,27,28]. In contrast with these common beliefs, the present study demonstrates that RyR2 from a number of species is phosphorylated by PKA at two major sites, Ser-2030 and Ser-2808, and that the phosphorylation of Ser-2030 does not reflect the phosphorylation status of RyR2 by PKA. We have shown that the phosphorylation of Ser-2030 is closely correlated with β-adrenergic stimulation and PKA activation, whereas Ser-2808 is substantially phosphorylated in the absence of β-adrenergic stimulation. Furthermore, both the basal and isoproterenol-induced phosphorylation of Ser-2808 is not significantly inhibited by PKI. Importantly, the phosphorylation of Ser-2030, but not of Ser-2808, is markedly enhanced in failing rat hearts in vivo in response to isoproterenol stimulation. These data indicate that Ser-2030, but not Ser-2808, is the major phosphorylation site in RyR2 that responds to PKA activation upon β-adrenergic stimulation in both normal and failing hearts.

**Ser-2030 is a conserved PKA phosphorylation site in RyR2**

We have previously shown that mutating Ser-2030 to alanine in recombinant mouse RyR2 does not abolish phosphorylation of RyR2 by PKA. This observation led to the identification of a novel PKA phosphorylation site, Ser-2030 [13]. However, it was recently reported that RyR2 isolated from mutant mice in which the Ser-2030 site has been mutated to alanine (S2030A) was no longer phosphorylated by PKA, suggesting that Ser-2030 is the only PKA phosphorylation site in native mouse RyR2 [10,11]. These observations raised concern that the recombinant mouse RyR2 that we have cloned and expressed in HEK293 cells may behave differently from native mouse RyR2 with respect to PKA phosphorylation. To address this concern, we isolated RyR2 from mouse hearts by immunoprecipitation. Using an anti-Ser-2030(PO\(_3\)) phospho-specific antibody, we found that native mouse RyR2, like recombinant mouse RyR2, was phosphorylated by PKA at Ser-2030 (Figure 1). We have extended these phosphorylation studies to RyR2 from other species, and found that both recombinant and native human and rabbit RyR2s were also phosphorylated by PKA at the residue corresponding to Ser-2030 of mouse RyR2 (Figure 2). We have previously shown that Ser-2030 is phosphorylated in native rat and canine RyR2 [13]. Taken together, the results of our previous and current studies indicate that Ser-2030 or its equivalent is a highly conserved PKA phosphorylation site in RyR2. It is not clear why phosphorylation was not detected in RyR2 isolated from the S2030A mutant mouse or the S2080A mutant human RyR2 in the studies of Marks et al. [7,10,11]. The failure to detect phosphorylation of RyR2 by PKA may result from a low efficiency of PKA phosphorylation and/or a low sensitivity of detection of phosphorylation. Differences in experimental conditions and in the RyR2 cDNA clones used may also contribute to this controversy.

**Phosphorylation of RyR2 at Ser-2030 does not represent the phosphorylation status of RyR2 by PKA**

The phosphorylation of Ser-2030 has been commonly used as an index for RyR2 phosphorylation by PKA [7,10,11,27,28]. However, several lines of evidence suggest that this practice is inappropriate. (i) Ser-2030 can be phosphorylated by multiple protein kinases, including PKA, CaMKII and PKG [17] (Figure 5). Hence, the phosphorylation level of Ser-2030 is determined not only by the activity of PKA, but also by the activity of other kinases, such as CaMKII and PKG. (ii) Both native and recombinant RyR2s from a number of species are substantially phosphorylated at rest (Figure 2). Up to 30–70% of the Ser-2030 phosphorylation sites are phosphorylated in RyR2 isolated from canine SR membranes that have not been treated with exogenous kinases (results not shown). Importantly, this basal phosphorylation of Ser-2030 is insensitive to inhibitors of PKA, CaMKII and PKG, but is sensitive to the non-specific kinase inhibitor, staurosporine (Figure 5B), suggesting that a kinase(s) other than PKA, CaMKII or PKG is responsible for the basal phosphorylation of Ser-2030. (iii) The basal phosphorylation of Ser-2030 is sensitive to the phosphatase inhibitor, calyculin A (Figure 6), indicating that the level of Ser-2030 phosphorylation is also influenced by phosphatases. Therefore the level of phosphorylation at Ser-2030 is substantially independent of PKA activity and is likely to be influenced by other kinases and phosphatases that have yet to be identified. On the other hand, very weak phosphorylation of Ser-2030 was detected at rest. Ser-2030 is strongly phosphorylated by PKA, but not by CaMKII [13], and is weakly phosphorylated by PKG compared with PKA (Figure 5). Hence, Ser-2030 is a more specific PKA phosphorylation site than Ser-2808. Taken together, these observations indicate that the phosphorylation of Ser-2030 does not represent the phosphorylation status of RyR2 by PKA. Instead, the phosphorylation of Ser-2030 is a more appropriate index for RyR2 phosphorylation by PKA.

**Ser-2030, but not Ser-2808, is the major phosphorylation site in RyR2 that mediates the impact of PKA activation upon β-adrenergic stimulation**

Ser-2030 and Ser-2808 differ not only in their basal level of phosphorylation, but also in their response to β-adrenergic stimulation. Ser-2030 was phosphorylated in response to isoproterenol stimulation in a concentration- and time-dependent manner, whereas Ser-2808 phosphorylation did not closely correlate with β-adrenergic stimulation (Figure 3). Ser-2808 was already significantly phosphorylated before β-adrenergic stimulation, and
the extent of the increase in Ser-2808 phosphorylation after β-
adrenergic stimulation was much less than that for Ser-2030. Im-
portantly, isoproterenol-induced phosphorylation of Ser-2030 was
markedly inhibited by PKI, whereas isoproterenol-induced phos-
phorylation of Ser-2808 was insensitive to PKI. These observa-
tions indicate that during β-adrenergic receptor activation, the
phosphorylation of Ser-2030 is primarily mediated by PKA, whereas
the phosphorylation of Ser-2808 is largely mediated by a kinase(s) other than PKA. In addition, Ser-2030 and Ser-2808 differ in their response to sustained β-adrenergic stimulation. Prolonged β-adrenergic activation caused a transient increase in Ser-2030 phosphorylation, but a sustained increase in Ser-2808 phosphorylation. It has been shown that prolonged β-adrenergic stimulation resulted in transient activation of the cAMP/PKA signalling pathway, but sustained activation of the CaMKII sig-
alling pathway [29]. Thus the transient nature of Ser-2030 phosphorylation suggests that Ser-2030 is phosphorylated by PKA. Whether the sustained phosphorylation of Ser-2808 during prolonged β-adrenergic stimulation is mediated by CaMKII has yet to be determined. Taken together, these observations indi-
cate that Ser-2030, but not Ser-2808, is the major PKA phos-
phorylation site in RyR2 that responds to PKA activation upon
β-adrenergic receptor stimulation, in contrast with the common belief that Ser-2808 mediates the action of PKA upon β-
adrenergic stimulation.

RyR2 is not hyperphosphorylated by PKA in heart failure

The issue regarding the phosphorylation status of RyR2 by PKA under different cardiac conditions is highly controversial. This
may be due, in part, to the lack of specific probes for assessing
the phosphorylation of RyR2 by PKA. Based on the belief that
Ser-2030 is the only PKA phosphorylation site in RyR2, the
phosphorylation of Ser-2808 has been widely used to reflect
the phosphorylation status of RyR2 by PKA. However, as we
have demonstrated in the present study, a substantial level of Ser-
2808 phosphorylation is unrelated to PKA activity, both at rest
and upon β-adrenergic stimulation. A more appropriate probe
for the phosphorylation of RyR2 by PKA, we believe, is the
phosphorylation of Ser-2030, since it is closely correlated with
β-adrenergic stimulation. Based on this reasoning, we determined
the phosphorylation status of Ser-2030 at Ser-2030 in non-failing
and failing rat hearts. We found very low-level phosphorylation of Ser-
2030 in both non-failing and failing rat hearts (Figures 7 and 9),
results that are similar to those derived from a canine model of
HF [13]. Taken together, these observations indicate that RyR2 is
not hyperphosphorylated by PKA in HF.

Our assessments of RyR2 phosphorylation by PKA, how-
ever, do not exclude the possibility that RyR2 may be hyper-
phosphorylated by kinases other than PKA in HF. We found
that the phosphorylation of Ser-2030 was significantly increased in
failing rat hearts compared with non-failing hearts. On the other
hand, the phosphorylation of Ser-2808 was similar in non-
failings and failing canine hearts [13]. Thus unlike Ser-2030 phos-
phorylation, the level of phosphorylation of RyR2 at Ser-2808
varies in different models of HF. The cause of this variation in
the amount of Ser-2030 phosphorylation in HF has yet to be de-
finied, but it is unlikely to be due to PKA. Because Ser-2808 can
be phosphorylated by multiple kinases, the increased Ser-2030
phosphorylation in HF may result from the increased activity of
one or more of these kinases. It has been shown that CaMKII
activity is increased upon sustained β-adrenergic stimulation and
in HF [29,30]. Thus it is possible that the increase in Ser-2808
phosphorylation observed in certain models of HF may result from
an increase in CaMKII activity, but not in PKA activity. In line

with this view, it has recently been shown that the phosphorylation
of RyR2 by CaMKII was increased in a rabbit model of HF [31].
Alternatively, since the basal phosphorylation level of Ser-2808
is regulated by protein phosphatases (Figure 6), the change in
the phosphorylation level of Ser-2808 may result from altered
phosphatase activities.

Phosphorylation of Ser-2030 by PKA and its relevance
to cardiac arrhythmia in heart failure

Although the β-adrenergic signalling pathway is down-regulated
in HF due to a chronically elevated level of catecholamines, a residual level of β-adrenergic responsiveness is preserved and is
believed to play an important role in triggering cardiac arrhythmia
in HF under stress conditions [32]. However, the molecular mech-
anism by which activation of the residual β-adrenergic receptors
increases the propensity for cardiac arrhythmia in HF is not well
understood. Consistent with the existence of residual β-adrenergic
responsiveness, we have recently shown that isoproterenol stim-
ulation markedly enhanced spontaneous contractile waves in
trabeculae isolated from rats with HF [23]. We have further
demonstrated in the present study that isoproterenol stimulation
markedly increased RyR2 phosphorylation at Ser-2030, but not at
Ser-2808, in failing rat hearts (Figure 9). These data suggest
that the phosphorylation of Ser-2030, but not of Ser-2808, may be
a critical event that leads to increased spontaneous Ca2+
waves, and triggered arrhythmia, upon stimulation of the residual β-
adrenergic activity in HF.

Conclusions

In summary, the present study reveals that the phosphorylation
of RyR2 at Ser-2030 both at rest and upon β-adrenergic stimu-
lation is largely independent of PKA. Thus the level of Ser-2030
phosphorylation does not reflect the phosphorylation status of
RyR2 by PKA. On the other hand, Ser-2030 is a highly conserved
PKA phosphorylation site in RyR2, and the phosphorylation of
Ser-2030 responds to β-adrenergic stimulation in a concentration-
time- and PKA-dependent manner. Furthermore, RyR2 is not
hyperphosphorylated by PKA in HF. Importantly, the phos-
phorylation of Ser-2030, but not of Ser-2808, responds strongly to
isoproterenol stimulation in rats with HF. Our results indicate that
Ser-2030, but not Ser-2808, is the major PKA phosphorylation site
responding to PKA activation upon β-adrenergic stimulation in normal and failing hearts, and suggest that the phosphorylation of
Ser-2030 may be an important mediator of altered Ca2+ handling
and triggered arrhythmia in HF.

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