Functional Consequence of Protein Kinase A-dependent Phosphorylation of the Cardiac Ryanodine Receptor

SENSITIZATION OF STORE OVERLOAD-INDUCED Ca\(^{2+}\) RELEASE*

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The phosphorylation of the cardiac Ca\(^{2+}\)-release channel (ryanodine receptor, RyR2) by protein kinase A (PKA) has been extensively characterized, but its functional consequence remains poorly defined and controversial. We have previously shown that RyR2 is phosphorylated by PKA at two major sites, serine 2030 and serine 2808, of which Ser-2030 is the major PKA site responding to β-adrenergic stimulation. Here we investigated the effect of the phosphorylation of RyR2 by PKA on the properties of single channels and on spontaneous Ca\(^{2+}\) release during sarcoplasmic reticulum Ca\(^{2+}\) overload, a process we have referred to as store overload-induced Ca\(^{2+}\) release (SOICR). We found that PKA activated single RyR2 channels in the presence, but not in the absence, of luminal Ca\(^{2+}\). On the other hand, PKA had no marked effect on the sensitivity of the RyR2 channel to activation by cytosolic Ca\(^{2+}\). Importantly, the S2030A mutation, but not mutations of Ser-2808, diminished the effect of PKA on RyR2. Furthermore, a phosphomimetic mutation, S2030D, potentiated the response of RyR2 to luminal Ca\(^{2+}\) and enhanced the propensity for SOICR in HEK293 cells. In intact rat ventricular myocytes, the activation of PKA by isoproterenol reduced the amplitude and increased the frequency of SOICR. Confocal line-scanning fluorescence microscopy further revealed that the activation of PKA by isoproterenol increased the rate of Ca\(^{2+}\) release and the propagation velocity of spontaneous Ca\(^{2+}\) waves, despite reduced wave amplitude and resting cytosolic Ca\(^{2+}\). Collectively, our data indicate that PKA-dependent phosphorylation enhances the response of RyR2 to luminal Ca\(^{2+}\) and reduces the threshold for SOICR and that this effect of PKA is largely mediated by phosphorylation at Ser-2030.

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Ventricular tachycardia (VT) is the leading cause of sudden death, particularly in patients with heart failure (HF), but the molecular mechanisms underlying the high incidence of VT in HF are not completely understood (1). A major cause of VT is believed to be delayed afterdepolarizations, which are produced by spontaneous Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) via the cardiac ryanodine receptor (RyR2) during SR Ca\(^{2+}\) overload (2–5), a process we referred to as store overload-induced Ca\(^{2+}\) release (SOICR) (6, 7). Physical or emotional stresses, which activate the β-adrenergic receptor (AR)/protein kinase A (PKA) signaling pathway, are common triggers for SOICR. The activation of PKA leads to the phosphorylation of several key Ca\(^{2+}\) handling proteins, including the L-type Ca\(^{2+}\) channel, phospholamban (PLB), and RyR2. The outcome of this PKA activation is an increase in the SR Ca\(^{2+}\) load, SR Ca\(^{2+}\) release, and consequently cardiac output (8). Although the impact of PKA phosphorylation of the L-type Ca\(^{2+}\) channel and PLB on Ca\(^{2+}\) influx and SR Ca\(^{2+}\) uptake is well understood, the functional consequence and physiological significance of the phosphorylation of RyR2 by PKA remains unclear and controversial (9).

Marks’ group has shown that RyR2 is phosphorylated by PKA at a single residue, Ser-2808 (10, 11), which was originally identified as a unique Ca\(^{2+}\)- and calmodulin-dependent protein kinase II phosphorylation site (12, 13). They propose that in HF this residue is hyperphosphorylated by PKA, resulting in the dissociation of a 12.6-kDa FK506-binding protein (FKBP12.6) from RyR2 and consequently increasing the sensitivity of the channel to activation by cytosolic Ca\(^{2+}\) and enhancing SR Ca\(^{2+}\) leak. However, we have recently shown that RyR2 is phosphorylated by PKA at two major sites, Ser-2030 and Ser-2808, of which Ser-2030 is the major site responding to PKA activation upon β-adrenergic stimulation, and that there is no hyperphosphorylation of RyR2 by PKA in canine HF (14). Jiang et al. (15) have also demonstrated that there are no measurable differences in the phosphorylation level of RyR2 by PKA between failing and non-failing canine hearts. Moreover, stoichiometric phosphorylation of both native and recombinant RyR2 at Ser-2030...
2808 and mutations of Ser-2808 fail to dissociate FKBP12.6 from RyR2 (16, 17). Furthermore, neither mutations of Ser-2808, nor the dissociation of FKBP12.6 affected the properties of RyR2 channels (16, 18). Hence the mechanism by which PKA modulates RyR2 requires further investigation.

The results of most in vitro studies using isolated RyR2 and SR membrane vesicles indicate that the phosphorylation of RyR2 by PKA increases its open probability or its responsiveness to fast Ca\(^{2+}\) transients (10, 19–22). However, the functional impact of PKA-dependent phosphorylation of RyR2 on SR Ca\(^{2+}\) release in intact cardiac myocytes is unclear and difficult to study. This is because SR Ca\(^{2+}\) release is dependent not only on the activity of RyR2, but also on the activity of the L-type Ca\(^{2+}\) channel and SR Ca\(^{2+}\) pump, both of which are also regulated by PKA (8). Studies of the properties of Ca\(^{2+}\) sparks and SR Ca\(^{2+}\) release have shown that the phosphorylation of RyR2 by PKA does not appear to produce an appreciable effect on SR Ca\(^{2+}\) release and that the PKA-induced augmentation of SR Ca\(^{2+}\) release is primarily due to increased Ca\(^{2+}\) influx and SR Ca\(^{2+}\) load (23, 24). These results are seemingly inconsistent with those of in vitro studies. The reasons for this apparent discrepancy are unknown.

Moderate modulation of RyR2 activity has been shown to have no sustained effect on stimulated SR Ca\(^{2+}\) release due to the regulation of RyR2 by luminal Ca\(^{2+}\), a phenomenon often referred to as “SR auto-regulation” (25), but it does exert a sustained impact on spontaneous Ca\(^{2+}\) release or SOICR (26). Hence, one may be able to study the modulation of RyR2 by examining the SOICR activity, rather than stimulated SR Ca\(^{2+}\) release or Ca\(^{2+}\)-induced Ca\(^{2+}\) release. Using this approach, we have recently shown that those RyR2 mutations that are linked to ventricular tachycardia and sudden death reduce the threshold for SOICR by sensitizing the channel to luminal Ca\(^{2+}\) activation (6, 7). In the present study, we assessed the effect of PKA activation on SOICR in rat ventricular myocytes. Our results indicate that PKA phosphorylation activates the RyR2 channel and consequently enhances the propensity for SOICR. These findings provide new insight into the arrhythmogenic mechanism of stress-induced cardiac arrhythmias.

**EXPERIMENTAL PROCEDURES**

**DNA Transfection and Preparation of Cell Lysate from HEK293 Cells**—HEK293 cells grown on 100-mm tissue culture plates were transfected with RyR2 wild-type (wt) or mutant RyR cDNA using Ca\(^{2+}\) phosphate precipitation as described previously (27). After transfection for 24–26 h, the cells were washed three times with PBS (137 mM NaCl, 8 mM Na\(_2\)HPO\(_4\), 1.5 mM KH\(_2\)PO\(_4\), 2.7 mM KCl) plus 2.5 mM EDTA and harvested in the same solution by centrifugation for 8 min at 700 × g in an International Electrotechnical Commission Centra-CL2 centrifuge. The cells were washed with PBS without EDTA and centrifuged again at 700 × g for 8 min. The PBS-washed cells were solubilized in a lysis buffer containing 25 mM Tris/50 mM NaCl, 1% CHAPS, 0.5% soybean phosphatidylcholine, 2.5 mM 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 phenylmethylsulfonyl fluoride). The mixture was incubated on ice for 1 h. Cell lysate was obtained by centrifugation at 16,000 × g for 30 min twice in a microcentrifuge at 4 °C to remove the unsolubilized materials.

**Generation of Stable, Inducible HEK293 Cell Lines**—The mutations S2030A, S2030D, S2808A, S2808D, and S2030A/S2808A (double mutation) were introduced into the mouse RyR2 by the overlap extension method using the polymerase chain reaction as described previously (14, 28). Stable, inducible HEK293 cell lines expressing the S2030D and S2030A mutants were generated using the Flp-In T-Rex Core Kit from Invitrogen.Briefly, Flp-In T-Rex-293 cells were cotransfected with the inducible expression vector pcDNA5/FRT/TO containing the mutant cDNAs and the pOG44 vector encoding the Flp recombinase in 1:5 ratios using the Ca\(^{2+}\) phosphate precipitation method. The transfected cells were washed with PBS (137 mM NaCl, 8 mM Na\(_2\)HPO\(_4\), 1.5 mM KH\(_2\)PO\(_4\), and 2.7 mM KCl) 24 h after transfection followed by a change into fresh media for 24 h. The cells were then washed again with PBS, harvested, and plated onto new dishes. After the cells had attached (~4 h), the growth medium was replaced with a selection medium containing 200 μg/ml hygromycin (Invitrogen). The selection medium was changed every 3–4 days until the desired number of cells was grown. The hygromycin-resistant cells were pooled, aliquoted, and stored at −80 °C. These positive cells are believed to be isogenic, because the integration of RyR2 cDNA is mediated by the Flp recombinase at a single FRT site. Each HEK293 cell line was tested for RyR2 expression using Western blotting analysis and immunocytomofluorescence staining (data not shown).

**Isolation of Adult Rat Ventricular Myocytes**—All studies with rats were approved by the Animal Care Committee 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 (NIH Publication no. 85-23, revised 1996). Single rat ventricular myocytes were isolated as described previously (29). Isolated cells were stored at room temperature in a solution containing 20 mM taurine, 5 mg/ml albumin, and 0.5 mM CaCl\(_2\), until used for single cell Ca\(^{2+}\) imaging studies.

**Single Cell Epifluorescent Ca\(^{2+}\) Imaging of Rat Ventricular Myocytes**—Freshly isolated rat ventricular myocytes were placed on glass coverslips coated with 0.02% (w/v) gelatin and 10 μg/ml fibronectin, and loaded with 5 μM fluo-4-AM Ca\(^{2+}\) (Molecular Probes) plus 0.02% pluronic F-127 dye in Krebs-Ringer-Hepes (KRH) buffer (125 mM NaCl, 5 mM KCl, 6 mM glucose, 1.2 mM MgCl\(_2\), and 25 mM Hepes, pH 7.4) (without KH\(_2\)PO\(_4\)) in the presence of 1.0 mM CaCl\(_2\) for 20 min at room temperature (22 °C). The coverslips were mounted in a perfusion chamber on an inverted microscope (Nikon TE2000-S) equipped with a S-Fluo 20×/0.75 objective. The [Ca\(^{2+}\)] was then stepped to 3 mM for 5 min before further increasing it to 6 mM. The cells were then continuously perfused with KRH buffer containing 6 mM CaCl\(_2\) at room temperature (22 °C) in the absence (5 min) and presence of 100 nM isoproterenol (Iso) (6–7 min). To minimize photo-induced damage, the cells were not excited during the initial 3.5 min of the control period (6 mM CaCl\(_2\) with no Iso) and the initial 1.5 min of Iso treatment. Time-lapse images were captured every 0.15 s during the excitation periods, using Compix Inc. Simple PCI 6 software. The images were analyzed to determine the
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fluo-4 signal for each Ca\(^{2+}\) wave. To capture the wave front of each Ca\(^{2+}\) wave, a series (10–15) of regions of interest, each 1 pixel-wide (<1 \(\mu\)m of the cell length), were created parallel to the Ca\(^{2+}\) wave front. These regions of interest were positioned so that the wave front would fall within one of these regions for each Ca\(^{2+}\) wave. The region of interest that displayed the highest fluo-4 signal at each time point was then used as the signal for that time point, thereby capturing every wave front. This method of analysis allowed for the measurement of the Ca\(^{2+}\) signal over a very small distance within the cell and capturing the wave front of each Ca\(^{2+}\) wave with a relatively slow recording speed (0.15 s per image).

Confocal Line-scan Fluorescence Ca\(^{2+}\) Imaging—Freshly isolated rat ventricular myocytes were loaded with the Ca\(^{2+}\) indicator dye fluo-4-AM (5 \(\mu\)M, 10 min), and line-scan Ca\(^{2+}\) images were acquired using a confocal microscope (LSM510, Zeiss) equipped with a 40×, 1.3 numerical aperture, oil-immersion objective. For Ca\(^{2+}\) wave image acquisition, the scanning line was positioned along the length of the cell with a line-scan rate of 10 ms/line. For paired comparison, images before and after Iso (100 nM) treatment were acquired from the same cells. All experiments were performed at room temperature. The slope of the leading front of a propagating wave in the line-scan (x – t) image was fitted with a linear function to measure the wave velocity. The wave front was then aligned according to the linear fitting and spatially averaged for time course and amplitude analysis. All image-processing programs were coded in interactive data language.

Single Channel Recordings in Planar Lipid Bilayers—Recombinant RyR2 wt treated with active- or inactive (boiled)-PKA and RyR2 mutants were partially purified from cell lysate by sucrose density gradient centrifugation (6). Heart phosphatidylethanolamine and brain phosphatidylserine (Avanti Polar Lipid), dissolved in chloroform, were combined in a 1:1 ratio (w/w), dried under nitrogen gas, and suspended in 30 \(\mu\)l of n-decane at a concentration of 12 mg lipid/ml. Bilayers were formed across a 250-\(\mu\)m hole in a Delrin partition separating two chambers. The cis chamber (800 \(\mu\)l) was connected to the head stage input of an Axopatch 200A amplifier (Axon Instruments Inc.). The trans chamber (1.2 ml) was held at virtual ground. A symmetrical solution containing 250 mM KCl and 25 mM Hepes (pH 7.4), was used for all recordings, unless indicated otherwise. A 4-\(\mu\)l aliquot (~1 \(\mu\)g of protein) of the sucrose density gradient-purified recombinant wt or mutant RyR2 protein was added to the trans chamber. Spontaneous channel activity was always tested for sensitivity to EGTA and Ca\(^{2+}\). The chamber to which the addition of EGTA inhibited the activity of the incorporated channel was presumed to correspond to the cytoplasmic side of the channel. The direction of single channel currents was always measured from the luminal to the cytoplasmic side of the channel, unless mentioned otherwise. Recordings were filtered at 2500 Hz. Free Ca\(^{2+}\) concentrations were calculated using the computer program of Fabiato and Fabiato (30). Data analyses were carried out using the pClamp 8.1 software (Axon Instruments Inc.).

![FIGURE 1. Effect of PKA on single RyR2 channels.](image)

**RESULTS**

**Activation of Single RyR2 Channels by PKA Requires Luminal Ca\(^{2+}\)—** An increasing body of evidence suggests that SR luminal Ca\(^{2+}\) plays an important role in the regulation of RyR2. To determine whether luminal Ca\(^{2+}\) is involved in the activation of RyR2 by PKA, we assessed the activity of single RyR2 channels before and after PKA treatment in the presence or absence of luminal Ca\(^{2+}\) using single channel recordings in planar lipid bilayers. As shown in Fig. 1, a single RyR2 wt channel displayed little activity before PKA treatment in the presence of 2.5 mM luminal Ca\(^{2+}\). The subsequent addition of PKA activated the RyR2 channel (Fig. 1A). On average, PKA increases the open probability \((P_o)\) of single RyR2 (wt) channels to 16.6 ± 3.7 times (mean ± S.E., n = 15, p < 0.002) that before PKA treatment (control). On the other hand, PKA pretreated with cAMP-dependent protein kinase inhibitor had no effect (1.11 ± 0.07
times that of the control, \( n = 4, p = 0.210 \) (Fig. 2C). Importantly, the stimulatory effect of PKA on RyR2 requires luminal Ca\(^{2+}\). In the presence of low luminal Ca\(^{2+}\) (3.6 \( \mu \)M), PKA did not significantly affect the activity of single RyR2 channels (Fig. 1B). The average \( P_o \) after PKA treatment at 2–4 \( \mu \)M luminal Ca\(^{2+}\) was 0.99 ± 0.07 times that of the control (\( n = 7, p = 0.876 \)) (Fig. 2C).

**Activation of Single RyR2 Channels by PKA Is Largely Mediated by Ser-2030**—We have previously shown that PKA phosphorylates RyR2 at two major sites, Ser-2030 and Ser-2808 (14). To dissect whether Ser-2030 or Ser-2808 or both mediates the activation of RyR2 by PKA, we assessed the effect of PKA on the single channel activities of the RyR2 mutants S2030A, S2080A, S2080D, and S2030A/S2808A. We used the S2080D mutation to mimic constitutive phosphorylation of RyR2 at Ser-2808, because Ser-2808 is already highly phosphorylated at rest (14, 22, 28). Single channel recordings of these mutant channels were carried out in the presence of 2.5 mM luminal Ca\(^{2+}\). As shown in Fig. 2A, PKA did not significantly affect the activity of the S2030A mutant. The average \( P_o \) after PKA treatment was 1.33 ± 0.29 times (\( n = 7, p = 0.276 \)) that of the control (Fig. 2C). Similarly, no significant PKA-induced increase in \( P_o \) was observed with single S2030A/S2808A mutant channels (1.16 ± 0.19 times, \( n = 9, p = 0.423 \)) (Fig. 2C). On the other hand, single S2080A and S2080D mutant channels were significantly activated by PKA. The average \( P_o \) values after PKA treatment are 11.1 ± 4.4 times (\( n = 10, p < 0.05 \)) that of the control for S2080A and 19.1 ± 5.9 times (\( n = 6, p < 0.02 \)) that of the control for S2080D (Fig. 2, B and C). These data demonstrate that Ser-2030 is a major PKA site that mediates the activating effect of PKA on RyR2.

**Phosphomimetic Mutation S2030D Enhances the Response of RyR2 to Luminal Ca\(^{2+}\) and the Propensity for SOICR**—To further investigate the role of Ser-2030 in the activation of RyR2 by luminal Ca\(^{2+}\), we assessed the impact of the S2030D and S2030A mutations, which we used to mimic constitutive phosphorylation of Ser-2030 and its abolition, respectively, on the response of RyR2 to a wide range of luminal Ca\(^{2+}\) concentrations. As shown in Fig. 3, both the S2030D and S2030A mutants exhibited little activity in the presence of low concentrations of cytosolic (45 nM) and luminal (45 nM) Ca\(^{2+}\) (Fig. 3, A and B). However, raising the luminal Ca\(^{2+}\) concentrations increased the \( P_o \) of single S2030D mutant channels much more markedly than that of single S2030A mutant channels (Fig. 3C). For instance, at 2.5 mM luminal Ca\(^{2+}\), the average \( P_o \) of single S2030D channels was 0.152 ± 0.041 (\( n = 10 \), significantly greater than that of single S2030A channels (0.017 ± 0.0097, \( n = 13 \)) (\( p < 0.01 \)). The response of single S2030A channels to luminal Ca\(^{2+}\) was similar to that of the wt (7). These data are consistent with the notion that PKA phosphorylation of RyR2 at Ser-2030 sensitizes the channel to luminal Ca\(^{2+}\).

Given the link between the luminal Ca\(^{2+}\) activation of RyR2 and SOICR, the enhanced response of the S2030D mutant to luminal Ca\(^{2+}\) should increase the propensity for SOICR. To test this possibility, we assessed the impact of the S2030D and S2030A mutations on SOICR in HEK293 cells. Stable, inducible HEK293 cells expressing the S2030D and S2030A mutants were loaded with fura-2-AM. SOICR was induced in these cells by elevating the external Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{o}\)) from 0.1 to 5 mM and monitored using a single cell Ca\(^{2+}\) epifluorescence-imaging technique. The propensity for SOICR was assessed by measuring the fraction of cells displaying Ca\(^{2+}\) oscillations at each [Ca\(^{2+}\)]\(_{o}\). Analyzing a total of 808 S2030D and 409 S2030A mutant cells revealed that a significantly higher fraction of the S2030D mutant cells exhibited Ca\(^{2+}\) oscillations at [Ca\(^{2+}\)]\(_{o}\) between 0.2 and 5.0 mM than did cells expressing the S2030A mutant (Fig. 3D) (\( p < 0.05 \)). These analyses also revealed that the S2030D mutant cells displayed a significantly higher frequency of Ca\(^{2+}\) oscillations (155.7 ± 5.7%) than did the S2030A mutant cells (\( p < 0.0001 \)). However, no significant difference in the amplitude of Ca\(^{2+}\) oscillations between the S2030D and S2030A mutant cells was detected (\( p = 0.469 \)). There were considerable variations in the amplitude of Ca\(^{2+}\) oscillations among individual HEK293 cells. The change in the amplitude as a result of the mutations may be too small to be revealed in the presence of those large cell-to-cell variations. Nevertheless,
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FIGURE 3. The S2030D mutation increases the luminal Ca$^{2+}$ response of single RyR2 channels. Single channel activities of the mutants S2030D (A) and S2030A (B) were recorded as described in the legend to Fig. 1. The Ca$^{2+}$ concentration on both the cytosolic and luminal faces of the channel was adjusted to 45 mM. The luminal Ca$^{2+}$ concentration was then increased to various levels by the addition of aliquots of CaCl$_2$ solution. The control single channel current traces for S2030D (A, panel a) and S2030A (B, panel a) are shown, while single channel current traces at 2.5 mM luminal Ca$^{2+}$ (panels b in A and B) are also depicted. The relationships between $P_o$ and luminal Ca$^{2+}$ concentration of single S2030D and S2030A mutant channels are shown in C. Stable, inducible HEK293 cells expressing S2030D or S2030A were grown on glass coverslips, induced with tetracycline for 16–18 h, and loaded with 5 μM fura-2-AM in KRH buffer for 20 min at room temperature. The cells were continuously perfused with KRH buffer with 0.1, 0.2, 0.3, 0.5, 1.0, 2.0, and 5.0 mM CaCl$_2$, or 5.0 mM CaCl$_2$, plus 5 mM caffeine. The fractions of S2030D and S2030A mutant cells that displayed Ca$^{2+}$ oscillations at various [Ca$^{2+}$]$_o$ are shown in D. Data shown are mean ± S.E. from 6–12 separate experiments.

FIGURE 4. Effect of PKA phosphorylation on the cytosolic Ca$^{2+}$ activation of RyR2. Single channel activities of the RyR2 wt treated with active PKA (solid circles) and inactive (boiled) PKA (open circles) were recorded in the presence of 45 mM luminal Ca$^{2+}$ and various concentrations of cytosolic Ca$^{2+}$. The relationships between open probability ($P_o$) and cytosolic Ca$^{2+}$ concentrations (pCa) are shown. Data points shown are individual measurements obtained from six PKA-treated and five boiled PKA-treated RyR2 channels.

these data indicate that the S2030D mutation increases the frequency and propensity for SOICR, which is consistent with its enhancing effect on the luminal Ca$^{2+}$ activation of RyR2.

Effect of PKA Phosphorylation on the Cytosolic Ca$^{2+}$ Activation of Single RyR2 Channels—We have previously shown that the S2030A and S2030D mutants displayed EC$_{50}$ values for Ca$^{2+}$ activation of $[^{3}H]$ryanodine binding virtually identical to that of RyR2(wt) (14). Because the EC$_{50}$ value of Ca$^{2+}$ activation of $[^{3}H]$ryanodine binding is similar to that of cytosolic Ca$^{2+}$ activation of single RyR2 channels (7), these observations indicate that the S2030A and S2030D mutations do not alter the sensitivity of RyR2 to activation by cytosolic Ca$^{2+}$. To directly determine whether phosphorylation of RyR2 by PKA affects the sensitivity of the channel to cytosolic Ca$^{2+}$, we examined the response of single RyR2 channels treated with active- or inactive (boiled)-PKA to a wide range of cytosolic Ca$^{2+}$ concentrations. We found that the EC$_{50}$ value for cytosolic Ca$^{2+}$ activation of the active PKA-treated RyR2 channels (0.14 μM) is similar to that of the boiled PKA-treated channels (0.18 μM) (Fig. 4). Taken together, these data indicate that the phosphorylation of RyR2 by PKA preferentially potentiates the luminal, but not the cytosolic, Ca$^{2+}$ response of the channel.

Activation of PKA Enhances the Propensity for SOICR in Rat Ventricular Myocytes—The impact of PKA-dependent phosphorylation of RyR2 on SR Ca$^{2+}$ release in cardiac myocytes has been difficult to define. This is because the modulation of the RyR2 activity will not by itself lead to a sustained effect on SR Ca$^{2+}$ release due to the auto-regulation of RyR2 by the SR luminal Ca$^{2+}$ content. On the other hand, the modulation of the RyR2 activity can lead to a sustained change in the magnitude of SOICR. Therefore, to understand the functional consequence of the phosphorylation of RyR2 by PKA at the cellular level, we assessed the effect of PKA activation on the properties of SOICR in freshly isolated rat ventricular myocytes. The cells were perfused with 6 mM [Ca$^{2+}$]$_o$ to induce SOICR, which was monitored using single cell Ca$^{2+}$ imaging. At 6 mM [Ca$^{2+}$]$_o$, spontaneous Ca$^{2+}$ waves were readily observed traveling along the cells. Fig. 5A shows typical SOICR in a single myocyte before and after the addition of 100 nM Iso to activate the β-adrenergic receptor/PKA signaling pathway.
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FIGURE 5. PKA activation reduces the threshold for SOICR in rat ventricular myocytes. Fluo-4-AM-loaded freshly isolated rat ventricular myocytes were perfused with 6 mM external Ca\(^{2+}\) in KRH. 100 nM isoproterenol was then applied to induce PKA phosphorylation of RyR2. The fluo-4 fluorescence signal was monitored using single cell Ca\(^{2+}\) imaging. A, representative fluorescence images every 33 frames in the absence (control) or presence of 100 nM isoproterenol (Iso) of a single ventricular myocyte. The fluo-4 fluorescence intensity of a representative cell before and after the addition of Iso is shown in B. The frequency and amplitude of Ca\(^{2+}\) oscillations before (control) and after Iso treatment are shown in C and D, respectively. Pooled data (mean ± S.E.) from 28 ventricular myocytes are shown (*, p < 0.05). E, fluo-4 fluorescent intensity of a representative rat ventricular myocyte responding to elevating extracellular Ca\(^{2+}\).

fluor-4 fluorescent intensities of a small region (1 pixel wide, <1 μm of the cell length) perpendicular to the longitudinal axis of the cell are shown. As seen in Fig. 5B, the addition of 100 nM Iso reduced the amplitude and increased the frequency of these Ca\(^{2+}\) waves. Iso stimulation also reduced the level of resting Ca\(^{2+}\) (Fig. 5B). Analyzing these Ca\(^{2+}\) waves in a number of cells before and after Iso stimulation revealed that the amplitude of Ca\(^{2+}\) waves was significantly reduced to 87 ± 2% that of the control (Fig. 5C), whereas the frequency of Ca\(^{2+}\) waves was significantly increased to 223 ± 11% (mean ± S.E., n = 28, p < 0.05) (Fig. 5D). These data suggest that the phosphorylation of RyR2 by PKA increases the propensity for SOICR in cardiocytes. Fig. 5E shows the response of a single cardiac cell to elevating extracellular Ca\(^{2+}\) with respect to SOICR. It has previously been shown by Eisner and colleagues (31) that elevating extracellular Ca\(^{2+}\) increases the SR Ca\(^{2+}\) content and that when the SR Ca\(^{2+}\) content reaches a threshold level, spontaneous Ca\(^{2+}\) release (SOICR) occurs. Once SOICR occurs, further increases in extracellular Ca\(^{2+}\) have no effect on the SR Ca\(^{2+}\) content or the amplitude of SOICR. As reported by Eisner and colleagues, we found that elevating extracellular Ca\(^{2+}\) markedly increased the frequency of Ca\(^{2+}\) waves but had no considerable effect on the amplitude of Ca\(^{2+}\) waves or the resting Ca\(^{2+}\) level (Fig. 5E).

Effect of PKA Activation on the Kinetic Properties of Ca\(^{2+}\) Waves in Rat Ventricular Myocytes—Iso stimulation also appeared to alter the spatiotemporal characteristics of the waves. The waveforms before and after Iso treatment seemed to be different (Fig. 5B). To more precisely determine the effect of PKA activation on the kinetics of SOICR, we employed a fast confocal line scanning fluorescence microscope. Freshly isolated rat ventricular myocytes were loaded with fluo-4-AM and perfused with 6 mM [Ca\(^{2+}\)]\(_o\) to induce SOICR. Ca\(^{2+}\) transients before and after the addition of 100 nM Iso were measured by confocal line scanning Ca\(^{2+}\) imaging. As shown in Fig. 6A, Ca\(^{2+}\) waves traveling along the cells were readily detected at elevated [Ca\(^{2+}\)]\(_o\) before and after the addition of Iso. As with those observed using the single cell epifluorescence imaging technique (Fig. 5), line-scanning confocal microscopy also revealed a significant decrease in the amplitude (8.6 ± 5.5%, p < 0.05) of spontaneous Ca\(^{2+}\) waves and the resting level of Ca\(^{2+}\) (27.8 ± 6.6%, p < 0.001) (n = 15) (Fig. 6, A, C, and D). Furthermore, kinetic analysis of the Ca\(^{2+}\) waves from a number of cells showed that Iso stimulation decreased the rising time (27.6 ± 7.3%, p < 0.001) and the half decay time (57.9 ± 6.7%, p <
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0.001), whereas it increased the rising rate (dF/dt, 30.9 ± 12.6%, p < 0.001) and the propagation velocity of the Ca^{2+} waves (40.5 ± 9.4%, p < 0.001) (n = 15) (Fig. 6, A, B, E, and F). The iso-induced reduction in the half decay time and resting Ca^{2+} level is likely due to the enhanced activity of the SR Ca^{2+}-ATPase as a result of PKA-dependent phosphorylation of phospholamban. On the other hand, the increases in the rate of Ca^{2+} release and the propagation velocity of the Ca^{2+} waves are likely the result of an iso-induced sensitization of SR Ca^{2+} release via the activation of RyR2. Hence, these kinetic analyses suggest that the phosphorylation of RyR2 by PKA in response to Iso stimulation enhances SOICR in rat ventricular myocytes.

DISCUSSION

Activation of the β-AR/PKA signaling pathway synchronizes Ca^{2+} mobilization and improves cardiac contractility in normal hearts. However, excessive β-AR stimulation often leads to delayed afterdepolarization-associated VT in diseased hearts (1). The molecular and cellular mechanisms underlying the arrhythmogenic nature of β-AR/PKA activation in diseased hearts, and particularly the role of PKA phosphorylation of RyR2 in this process are not completely understood. The focus of the present study is to understand the functional consequence of PKA-dependent phosphorylation of RyR2 at the molecular and cellular levels. Our data demonstrate that PKA phosphorylation activates the RyR2 channel and enhances the propensity for SOICR. Considering the link between SOICR and triggered arrhythmias, an enhanced propensity for SOICR as a result of PKA phosphorylation of RyR2 may account for the increased susceptibility to stress-induced cardiac arrhythmias in diseased hearts.

Activation of RyR2 by PKA Is Primarily Mediated by Phosphorylation at Ser-2030—Although the phosphorylation of RyR2 by PKA has been extensively investigated, the molecular mechanism by which PKA modulates the activity of RyR2 has remained unclear and controversial. Marks and his colleagues (11) claimed that RyR2 is phosphorylated by PKA at a single site Ser-2808, and that the phosphorylation of Ser-2808 dissociates FKB12.6 from RyR2, consequently inducing subconductance states and increasing the sensitivity of the channel to cytoplasmic Ca^{2+} (10). They also showed that mutating Ser-2808 to alanine (S2808A) abolished the impact of PKA, whereas mutating Ser-2808 to aspartate (S2808D), which mimics phosphorylation, resulted in a constitutive activation of RyR2. Furthermore, they have demonstrated that RyR2 isolated from knock-in mice harboring the S2808A mutation can no longer be phosphorylated by PKA and that these S2808A knock-in mice were protected against the development of heart failure. These observations have led them to conclude that Ser-2808 is the only PKA site on RyR2 that mediates the activation of RyR2 by PKA (11).

In contrast to these conclusions, Stange et al. (16) demonstrated that these mutations, S2808A and S2808D, have no effect on either FKB12.6-RyR2 association or the intrinsic properties of the RyR2 channel. We have likewise shown that neither mutations at Ser-2808 nor stoichiometric phosphorylation of Ser-2808 by PKA dissociate FKB12.6 from RyR2 (17). Recently, Valdivia’s group has also generated S2808A knock-in mice and demonstrated that the RyR2 protein isolated from S2808A knock-in mice could be phosphorylated by PKA at Ser-2030. Importantly, the S2808A mutation did not significantly alter the response of these mice to β-adrenergic stimulation. Furthermore, the Ser-2808 mutant mice were not significantly protected against the development of heart failure (32). Moreover, several groups have reported that RyR2 from non-stimulated, normal hearts is already substantially phosphorylated at Ser-2808 (up to 75%) (14, 16, 22, 28, 33). Single RyR2 channels phosphorylated at Ser-2808 to ~75% of maximum display a low P, and no subconductance states (22). These observations indicate that RyR2 from normal hearts is substantially phosphorylated at Ser-2808 and that hyperphosphorylation of Ser-2808 has no correlation with the occurrence of subconductance states or increased channel activity. Interestingly, PKA is still able to dramatically activate these highly Ser-2808-phosphorylated single RyR2 channels, again with no induction of subconductance states (22). One explanation for these observations is that the activation of RyR2 by PKA may require stoichiometric (100%) phosphorylation of RyR2 at Ser-2808. Therefore, despite intensive studies, the functional consequence and physiological role of Ser-2808 phosphorylation remain elusive. Clearly, more investigations are needed to resolve these issues. Nevertheless, these observations suggest that Ser-2808 is unlikely to be the sole phosphorylation site that mediates the PKA-dependent activation of RyR2 and that additional PKA sites are likely involved.

We have recently shown that RyR2 is phosphorylated by PKA at two major sites, Ser-2030 and Ser-2808 (14). More recently, we have demonstrated that Ser-2030, but not Ser-2808, is the major phosphorylation site in RyR2 that responds to PKA activation upon β-AR stimulation in both normal and failing hearts (28). In the present study, we have demonstrated that PKA is able to activate single RyR2 (wt), S2808A, and S2808D mutant channels, but not single S2030A mutant channels, indicating that the activation of RyR2 by PKA is largely mediated by phosphorylation of Ser-2030. These findings are consistent with the observation that mutations at Ser-2808 have little effect on the properties of the RyR2 channel (16). Our findings also provide an alternative explanation as to why single RyR2 channels already heavily phosphorylated at Ser-2808 (~75% of maximum) are still robustly and consistently activated by PKA (22). It is likely that the PKA-dependent activation of RyR2 observed in the study of Carter et al. is attributable, at least in part, to the phosphorylation of Ser-2030, which displays very little basal phosphorylation at rest.

PKA-dependent Phosphorylation of RyR2 Reduces the Threshold for SOICR—It is known that SOICR occurs when the SR luminal Ca^{2+} concentration reaches a threshold level as a result of the activation of RyR2 by luminal Ca^{2+}. The results of our single channel studies indicate that PKA phosphorylation potentiates the response of RyR2 to luminal Ca^{2+}. Interestingly, PKA-dependent phosphorylation of RyR2 does not considerably affect the response of the channel to activation by cytosolic Ca^{2+} (Fig. 4). This preferential potentiation of luminal Ca^{2+} activation of RyR2 by PKA would lead to enhanced SOICR. Consistent with this view, we found that the activation of PKA with Iso treatment reduced the amplitude and increased the
frequency of SOICR and hastened the rising and propagation of Ca\(^{2+}\) waves in rat ventricular myocytes (Figs. 5 and 6).

This reduction in the amplitude of SOICR is unlikely to be due to the effect of PKA on the L-type Ca\(^{2+}\) channel or the SERCA/PLB system. This is because PKA-dependent phosphorylation of the L-type Ca\(^{2+}\) channel and PLB are known to increase Ca\(^{2+}\) influx and SR Ca\(^{2+}\) uptake, both of which in turn enhance the magnitude of SR Ca\(^{2+}\) release. This effect of PKA on the L-type Ca\(^{2+}\) channel or the SERCA/PLB system is opposite to that observed with SOICR. Furthermore, during SR Ca\(^{2+}\) overload, SOICR occurs in the absence of membrane depolarization, during which the L-type Ca\(^{2+}\) channel would not be activated. Hence, the L-type Ca\(^{2+}\) channel is unlikely to be involved in spontaneous SR Ca\(^{2+}\) release or SOICR. SERCA activity is also unlikely to affect the magnitude of SOICR. This is because, during SR Ca\(^{2+}\) overload, the SR is maximally loaded with Ca\(^{2+}\). Changes in SERCA activity by PKA via the phosphorylation of PLB would only change the rate, but not the maximum level, of SR Ca\(^{2+}\) loading. On the other hand, the increase in the frequency of SOICR upon PKA activation is likely to result from the reduction in the SOICR threshold itself and the activation of the SERCA/PLB system. This is because a store with a reduced threshold will take less time to refill and an enhanced Ca\(^{2+}\) pumping activity will speed up its refilling, both of which would increase the frequency of SOICR. Hence, studying the magnitude of SOICR, rather than that of Ca\(^{2+}\)-induced Ca\(^{2+}\) release, is a more appropriate approach for assessing the activity of RyR2, as suggested by Eisner et al. (25).

Iso stimulation also reduced the level of resting cytosolic Ca\(^{2+}\) in rat ventricular myocytes as a result of increased SERCA activity (Fig. 6C). A reduced level of resting cytosolic Ca\(^{2+}\) would decrease the activity of RyR2. It has been shown that moderate inhibition of RyR2 activity increases the amplitude of SOICR, whereas modest stimulation of RyR2 decreases the SOICR threshold. Hence, the Iso-induced reduction in resting cytosolic Ca\(^{2+}\) would in turn increase the amplitude of SOICR. This indirect effect of PKA on RyR2 via resting cytosolic Ca\(^{2+}\) is opposite to the direct effect of PKA-dependent phosphorylation on RyR2. Therefore, the apparent reduction in the SOICR amplitude observed in rat ventricular myocytes after Iso treatment would have been greater if the level of resting cytosolic Ca\(^{2+}\) were maintained. In other words, the extent of the effect of PKA-dependent phosphorylation of RyR2 on the amplitude of SOICR may have been underestimated. In this regard, it will be of interest to determine the impact of Iso stimulation on SOICR in cardiac myocytes isolated from PLB-knock-out mice.

These effects of PKA on SOICR resemble those of low concentrations of caffeine, which has been shown to enhance the activity of RyR2 and reduce the SOICR threshold, thus reducing the amplitude of Ca\(^{2+}\) waves (6, 26). Moreover, treating cardiac cells with Iso led to a marked increase in the phosphorylation of RyR2 at Ser-2030 and, to a lesser extent, at Ser-2808 (28). Therefore, these data together suggest that, like caffeine, the phosphorylation of RyR2 by PKA reduces the threshold for SOICR in rat ventricular myocytes.

Because the activation of RyR2 by PKA requires luminal Ca\(^{2+}\), the impact of the phosphorylation of RyR2 by PKA may not be readily manifested depending on the Ca\(^{2+}\) content of the SR. Indeed, by controlling the levels of triggering Ca\(^{2+}\) and SR Ca\(^{2+}\) content, Li et al. (23) have demonstrated that the phosphorylation of RyR2 by PKA has no significant effect on the properties of Ca\(^{2+}\) sparks in permeabilized or intact mouse cardiac myocytes at rest. Further studies with controlled triggering Ca\(^{2+}\) and SR Ca\(^{2+}\) load have also revealed that the PKA-dependent phosphorylation of RyR2 has little effect on SR fractional Ca\(^{2+}\) release and EC coupling gain but accelerates the kinetics of SR Ca\(^{2+}\) release (24). It has been shown that the activity of RyR2 affects the SR Ca\(^{2+}\) content, which in turn negatively regulates the activity of RyR2 (25). It is possible that the impact of PKA-dependent phosphorylation of RyR2 is counteracted by subtle changes in the SR Ca\(^{2+}\) content, and hence may not be readily revealed if the SR is not maximally loaded. Nevertheless, these observations suggest that with a normal SR Ca\(^{2+}\) load PKA-dependent phosphorylation of RyR2 may play an important role in the kinetics and synchronization of SR Ca\(^{2+}\) release (24, 34, 35). In line with this view, we found that the activation of PKA by Iso hastened the rising and propagation of Ca\(^{2+}\) waves (Fig. 6).

Proposed Role for PKA-dependent Phosphorylation of RyR2 in Stress-induced Cardiac Arrhythmias—Obayashi et al. (36) have recently shown that spontaneous Ca\(^{2+}\) release in trabeculae from rats with HF is increased. This observation implies that the threshold for SOICR in failing hearts is reduced, which is consistent with the observation that single RyR2 channels isolated from failing canine hearts exhibited an increased sensitivity to luminal Ca\(^{2+}\) (37). Furthermore, we have shown that RyR2 in rats with HF was phosphorylated at Ser-2030 upon Iso injection. Based on these observations and the results of the present studies, we propose a model for the role of RyR2 in stress-induced cardiac arrhythmias in HF (Fig. 7). We hypothesize that the threshold for SOICR is primarily determined by the sensitivity of RyR2 to luminal Ca\(^{2+}\). In the normal SR, the threshold for SOICR is higher than the SR free Ca\(^{2+}\) level at rest. Under stressful conditions, PKA is activated, leading to an increase in the SR free Ca\(^{2+}\) level. Concomitantly, PKA-dependent phosphorylation of RyR2 reduces the threshold for SOICR. The reduced SOICR threshold, however, is still higher than the increased SR free Ca\(^{2+}\) level, so that there is little or no Ca\(^{2+}\) spillover from the normal SR in either the resting or stimulated states. On the other hand, in the abnormal (HF) SR, the threshold for SOICR is already reduced due to abnormal modulation of RyR2. Under resting conditions, the reduced threshold for SOICR is higher than the SR free Ca\(^{2+}\) level, so that there is little or no Ca\(^{2+}\) spillover from the HF SR. However, under stressful conditions, the HF SR is abruptly overloaded with Ca\(^{2+}\), and the already reduced threshold for SOICR is further decreased by PKA-dependent phosphorylation of RyR2. Because of the reduced threshold, SOICR is more likely to occur in the HF SR during SR Ca\(^{2+}\) loading. The resulting SR Ca\(^{2+}\) spillover can lead to delayed afterdepolarizations and triggered arrhythmia. Also because of the reduced threshold, the resting SR free Ca\(^{2+}\) in HF may adapt to a reduced level due to SR Ca\(^{2+}\) auto-regulation, which would account for the reduced SR Ca\(^{2+}\) content commonly observed in HF.

We have recently shown that RyR2 mutations associated with Catecholaminergic polymorphic VT and sudden death
reduce the threshold for SOICR by increasing the sensitivity of RyR2 to activation by luminal Ca\(^{2+}\) (6, 7). The effect of PKA-dependent phosphorylation of RyR2 resembles that of those RyR2 mutations. Thus, enhanced luminal Ca\(^{2+}\) activation of RyR2 and a reduced SOICR threshold may represent a common cause of stress-induced cardiac arrhythmias in patients with HF and VT.

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FIGURE 7. Proposed role of PKA-dependent phosphorylation of RyR2 in stress-induced arrhythmias. The relationship between the threshold for SOICR and the SR free Ca\(^{2+}\) level in normal (A) and abnormal SR (B) in the non-stimulated and stimulated states is schematically shown. The threshold for SOICR is depicted by a red bar. The SR free Ca\(^{2+}\) level is represented by the blue area. PKA activation leads to an abrupt increase in SR free Ca\(^{2+}\), which is depicted by the yellow area, and a reduction in the threshold for SOICR, as indicated by the red arrow. When the SR free Ca\(^{2+}\) level exceeds the SOICR threshold, SOICR occurs, leading to SR Ca\(^{2+}\) spillover, which in turn could generate delayed afterdepolarizations and triggered arrhythmia.