Protein Kinase A Phosphorylation of the Ryanodine Receptor Does Not Affect Calcium Sparks in Mouse Ventricular Myocytes

Yanxia Li, Evangelia G. Kranias, Gregory A. Mignery, Donald M. Bers

Abstract—Ryanodine receptor ( RyR ) phosphorylation by protein kinase A ( PKA ) may be important in modulating resting sarcoplasmic reticulum ( SR ) $\text{Ca}^{2+}$ release, especially in heart failure. However, clear cellular data on PKA-dependent modulation of cardiac RyRs is limited because of difficulty in distinguishing between PKA effects on RyR, phospholamban ( PLB ), and $\text{Ca}^{2+}$ current. To clarify this, we measured resting $\text{Ca}^{2+}$ sparks in permeabilized ventricular myocytes from wild-type ( WT ) and PLB knockout ( PLB-KO ) mice and transgenic mice expressing only double-mutant PLB ( PLB-DM ) that lacks the regulatory phosphorylation sites ( S16A/T17A ). In WT myocytes, cAMP dramatically increased $\text{Ca}^{2+}$ spark frequency ( CaSpF ) by 2- and 3-fold when $[\text{Ca}^{2+}]$ was clamped at 50 and 10 nmol/L ( and the SR $\text{Ca}^{2+}$ content also rose by 40% and 50% ). However, in PLB-KO and PLB-DM, neither CaSpF nor SR $\text{Ca}^{2+}$ load was changed by the addition of 10 μmol/L cAMP ( even with phosphatase inhibition ). PKA activation also increased $\text{Ca}^{2+}$ spark amplitude, duration, and width in WT, but not in PLB-KO or PLB-DM. RyR phosphorylation was confirmed by measurements of $^{32}$P incorporation on immunoprecipitated RyR. In intact resting myocytes, PKA activation increased CaSpF 2.8-fold in WT, but not in PLB-KO, confirming results in permeabilized myocytes. We conclude that the PKA-dependent increase in myocyte CaSpF and size is entirely attributable to PLB phosphorylation and consequent enhanced SR $\text{Ca}^{2+}$ load. PKA does not seem to have any appreciable effect on resting RyR function in these ventricular myocytes. Moreover, the data provide compelling evidence that elevated intra-SR $[\text{Ca}^{2+}]$ increases RyR gating independent of cytosolic $[\text{Ca}^{2+}]$ ( which was clamped ). ( Circ. Res. 2002;90:309-316. )

Key Words: cardiac muscle ■ excitation-contraction coupling ■ calcium transport ■ sarcoplasmic reticulum

Beta-adrenergic receptor ( β-AR ) activation regulates beat-to-beat cardiac function. β-AR activation via the sympathoadrenal system activates adenylyl cyclase via a GTP-binding protein ( Gs ) and produces cAMP. Activation of cAMP-dependent protein kinase ( PKA ) phosphorylates functional proteins in the sarcolemma ( notably $\text{Ca}^{2+}$ channels ), the sarcoplasmic reticulum ( SR ), and myofibrils ( troponin I and protein C ).

Phospholamban ( PLB ) and the SR $\text{Ca}^{2+}$ release channel/ryanodine receptor ( RyR ) are the key SR proteins phosphorylated by PKA in cardiac myocytes. PLB phosphorylation relieves its otherwise tonic inhibition of the SR $\text{Ca}^{2+}$-ATPase. This increases SR $\text{Ca}^{2+}$ uptake rate, accelerates intracellular $[\text{Ca}^{2+}]$ ( $[\text{Ca}^{2+}]_{i}$ ) decline during relaxation, and tends to increase SR $\text{Ca}^{2+}$ content. In addition, $\text{Ca}^{2+}$ current ($I_{\text{Ca}}$) is increased by PKA. This increases the $\text{Ca}^{2+}$ trigger for SR $\text{Ca}^{2+}$ release and also increases cellular and SR $\text{Ca}^{2+}$ content. PKA-dependent RyR phosphorylation has also been reported to increase the open probability ( P o ) of the RyR in lipid bilayers. Moreover, Marx et al reported RyR hyperphosphorylation ( at Ser-2809 ) in heart failure ( HF ), which caused release of FK-506 binding protein ( FKBP ) from the RyR and increased overall P o . There was an increased $\text{Ca}^{2+}$ sensitivity of RyR opening and reduced coupling of gating events. They also proposed that this could cause a diastolic SR $\text{Ca}^{2+}$ leak in HF leading to reduced SR $\text{Ca}^{2+}$ content and contractile dysfunction. SR $\text{Ca}^{2+}$ content is decreased in human, canine, and rabbit HF, but this has generally been attributed to reduced SR $\text{Ca}^{2+}$-ATPase or increased $\text{Na}^{+}$-$\text{Ca}^{2+}$ exchanger expression or function.

$\text{Ca}^{2+}$ sparks are the fundamental local SR $\text{Ca}^{2+}$ release events detected in intact ventricular myocytes. $\text{Ca}^{2+}$ sparks are probably due to a cluster of 6 to 20 RyRs acting in concert to produce both the resting diastolic SR $\text{Ca}^{2+}$ leak and the temporally synchronized SR $\text{Ca}^{2+}$ release during excitation-contraction ( E-C ) coupling, although individual $\text{Ca}^{2+}$ sparks during E-C coupling are obscured by temporal and spatial overlap.

Functional results about PKA effects on RyR are mostly from isolated systems ( eg, single-channel bilayer recordings ).
or SR vesicles). Attempts to study effects of PKA on the RyR in more intact cardiac myocytes are complicated by changes in $I_{Ca}$, SR Ca$^{2+}$-ATPase, SR Ca$^{2+}$ content, [Ca$^{2+}$]], and myofilament properties. Nevertheless, there are reports that suggest that PKA can either enhance or depress E-C coupling in intact cells.

In the present study, we minimize complicating factors to assess the impact of RyR PKA phosphorylation on resting SR Ca$^{2+}$ leak (Ca$^{2+}$ sparks) in a relatively intact cellular environment. Myocytes were permeabilized with streptolysin-O (SLO). This functionally removes I$_{Ca}$ and allows control of [Ca$^{2+}$], by exogenous Ca$^{2+}$ buffers and indicators. Furthermore, we used mice in which the PLB gene was knocked out (PLB-KO) and also in which a double-mutant nonphosphorylatable form of PLB was transgenically expressed in the knockout background (PLB-DM). The mutant PLB has alanine replacing both Ser-16 (PKA site) and Thr-17 (CaMKII site). These mouse models abolish the complicating effect of PKA on SR Ca$^{2+}$-ATPase activity.

We sought to determine how RyR function was altered by PKA phosphorylation in SLO-permeabilized ventricular myocytes. In wild-type (WT) myocytes, PKA activation increased SR Ca$^{2+}$ content and Ca$^{2+}$ spark frequency (CaSpF) and spark amplitude. However, in PLB-KO or PLB-DM myocytes, PKA activation had no effect on either CaSpF, spark amplitude, or SR Ca$^{2+}$ content (where RyR phosphorylation was confirmed). In intact myocytes, PKA activation also increased resting CaSpF in WT, but not in PLB-KO. We conclude that PKA phosphorylation of RyR has no significant effect on resting RyR-mediated SR Ca$^{2+}$ leak. In this light, the increase in CaSpF in WT myocytes shows that elevated SR Ca$^{2+}$ content directly increases CaSpF.

**Materials and Methods**

**Cardiac Myocyte Isolation**

Single mouse ventricular myocytes were isolated similar to previously described methods. Mice were handled according to the Council of the American Physiological Society. Some WT mice were superfused with relaxing solution containing (in mmol/L) NaCl 150, Tris-HCl 10, EGTA 1, MgCl$_2$ 5, phenylmethylsulfonyl fluoride, 1 mmol/L leupeptin, and 1 μg/mL aprotinin. After solubilization and removal of insoluble material, RyR was immunoprecipitated using mouse monoclonal anti-RyR IgG (MA3-925, Affinity Bioreagents Inc) and protein A Sepharose (Pharmacia Biotech AB). The protein A Sepharose was then washed 6 times with increasing [NaCl] (0 to 300 mmol/L). Phosphorylated protein was separated by SDS-PAGE (5%), dried, autoradiographed, and analyzed by PhosphorImager.

**Ca$^{2+}$ Spark Measurement in Intact Myocytes**

Intact mouse ventricular myocytes were loaded with 10 μmol/L fluo-4 AM for 20 minutes and washed for 15 minutes in normal

**Figure 1.** Fluo-3 calibration curve. Calibration solutions with [Ca$^{2+}$] ranging from 10 nmol/L to 10 μmol/L (with 20 μmol/L fluo-3) were measured on the confocal microscope. The Ca$^{2+}$-fluoride dissociation constant ($K_d$) was 700 nmol/L.

0.5, glutathione (reduced form) 10, 8% dextran (MW 40 000), and 50 μmol/L fluo-3 (K-salt). Thus, [Ca$^{2+}$], is clamped by [Ca$^{2+}$], in the permeabilized system.

**Ca$^{2+}$ Signal Recording Using Confocal Microscopy and Fluo-3**

Ca$^{2+}$ sparks were recorded by a laser scanning confocal microscope (LSM 410, Carl Zeiss) with a ×40 oil immersion objective (numeric aperture=1.3). Fluo-3 was excited at the 488-nm line of an argon laser with emission collected through a 515-nm long-pass filter. Fluorescence images were recorded in line-scan mode with 512 pixels per line at 250 Hz. In vitro calibration (Figure 1) was done using fluo-3 (K-salt) and a set of solutions with known [Ca$^{2+}$], ranging from 10 nmol/L to 10 mmol/L (calculated using MaxChela-tor). The $K_d$ was 700 nmol/L. Free [Ca$^{2+}$] in the experimental solutions was used confirmed by the in vitro calibration. Since [Ca$^{2+}$], at rest (Ca$^{2+}$,rest) and $K_d$ are known, [Ca$^{2+}$], was calculated:

$$[Ca^{2+}]_i = \frac{K_d F/F_0 (K_d/ [Ca^{2+}]_i)_{rest} + 1/F_0]}{1 + F/F_0}.$$  

SR Ca$^{2+}$ load was evaluated by the Ca$^{2+}$ transient induced by the application of 10 mmol/L caffeine. Because two different [Ca$^{2+}$], were used and [Ca$^{2+}$], was buffered differently, reliable Ca$^{2+}$ transient amplitude comparisons are best made at the same [Ca$^{2+}$].

**RyR Phosphorylation in SLO-Permeabilized Myocytes**

SLO-permeabilized myocytes were incubated in internal solution containing 1 μmol/L $[^{32}P]$ATP (150 μCi, New England Nuclear). To mimic Ca$^{2+}$ spark conditions, 10 μmol/L cAMP was added to some of the permeabilized cells (5 minutes, 23°C) in a similar solution used for Ca$^{2+}$ sparks, except that [ATP] was only 1 μmol/L (to maintain $^{32}$P specific activity). To minimize cellular RyR phosphorylation, some incubates included protein phosphatase type 1 (PP1, 20 U/mL; Sigma), 2 mmol/L MnCl$_2$ (to maximize PP1 activity), and protein kinase inhibitor (PKI, 150 μmol/L; Sigma). In another control to maximize RyR phosphorylation, we included protein phosphatase inhibitors (PPIs), okadaic acid (10 μmol/L; Sigma) and 2 mmol/L NaF with CAMP. Reactions were terminated by pelleting cells and adding solubilization buffer (in mmol/L): NaCl 150, Tris-HCl 10, EGTA 1, EDTA 1, mercaptoethanol 1.5, 1% Triton X-100, 2% BSA, and a mixture of protease inhibitors (1 μmol/L phenylmethylsulfonyl fluoride, 1 mmol/L leupeptin, and 1 μg/mL aprotinin). After solubilization and removal of insoluble material, RyR was immunoprecipitated using mouse monoclonal anti-RyR IgG (MA3-925, Affinity Bioreagents Inc) and protein A Sepharose (Pharmacia Biotech AB). The protein A Sepharose was then washed 6 times with increasing [NaCl] (0 to 300 mmol/L). Phosphorylated protein was separated by SDS-PAGE (5%), dried, autoradiographed, and analyzed by PhosphorImager.

**Table 3**

<table>
<thead>
<tr>
<th>Protein</th>
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<td>PKA</td>
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<td>Adenosine Triphosphate</td>
<td>Sigma</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
<td>Sigma</td>
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</table>

**Figure 2**

Calibration solutions with known [Ca$^{2+}$] were used to determine the Ca$^{2+}$ fluorescence by confocal microscopy. The Ca$^{2+}$-fluoride dissociation constant ($K_d$) was 700 nmol/L.
Tyrode’s (NT) solution containing (in mmol/L) NaCl 140, KCl 6, HEPES 10, glucose 10, MgCl₂ 1, and 1 or 2 mmol/L CaCl₂ (in PKB-KO and WT mouse myocytes, respectively, at 23°C). Isoproterenol (1 μmol/L) was added to stimulate β-AR. CaSpF was measured before and 3 minutes after isoproterenol addition (peak CaSpF in permeabilized WT).

Data Analysis

Ca²⁺ sparks were characterized using Interactive Data Language (IDL 5.3 computer software) and the algorithm of Cheng and colleagues²⁶,²⁷ and confirmed visually. Briefly, Ca²⁺ sparks are areas where fluorescence is 3.8 times the standard deviation (SD) of background fluorescence. Ca²⁺ spark peaks were normalized (F/F₀) to fluorescence baseline (F₀). Ca²⁺ spark duration was the full-duration half-maximum (FDHM) and width was the full-width half-maximum (FWHM). CaSpF was normalized to cell volume and depth (1 μm). SR Ca²⁺ load was evaluated by Ca²⁺ transient amplitude upon caffeine application.

Results are expressed as mean±SEM. Significance (P<0.05) was determined using Student’s t test for gaussian data and Mann-Whitney test otherwise.

Results

Ca²⁺ Spark Frequency in SLO-Permeabilized Myocytes

Figure 2A shows Ca²⁺ spark images from WT and PLB-KO mouse ventricular myocytes before and 3 minutes after addition of cAMP to stimulate PKA. In WT, cAMP clearly increased CaSpF, but not in PLB-KO. The same was true for WT with PLB-DM. That is, as shown in Figure 2B, CaSpF increased in WT, but not when the nonphosphorylatable PLB replaced native PLB.

Two different buffered resting [Ca²⁺]i, were used in the permeabilized cells (50 and 10 nmol/L). The higher value is somewhat below expected [Ca²⁺], in intact cells, but was chosen so that PKA-induced increases in CaSpF did not cause overlapping Ca²⁺ sparks (macro-sparks) or waves. Ca²⁺ waves were also limited by 0.5 mmol/L EGTA. Even at 50 nmol/L [Ca²⁺]i in permeabilized WT myocytes, resting CaSpF was higher (>350 sparks pl⁻¹ s⁻¹) than we typically observe in intact mouse myocytes (40 to 100 sparks pl⁻¹ s⁻¹) where we assume [Ca²⁺]i ≈100 nmol/L. For PLB-KO myocyte experiments, [Ca²⁺]i was even lower (10 nmol/L) for the same reason. That is, without PLB, the SR Ca²⁺-ATPase is very active and the CaSpF at 50 nmol/L [Ca²⁺]i was too high to confidently detect further increases. Thus, WT myocyte served as controls at each [Ca²⁺]i.

Figure 3 shows pooled data. In WT, addition of cAMP increased CaSpF dramatically, at both 10 nmol/L [Ca²⁺]i (Figure 3A) and 50 nmol/L [Ca²⁺]i (Figure 3B). In sharp contrast, there was no change in CaSpF for either PLB-KO or PLB-DM. Raising [Ca²⁺]i from 10 to 50 nmol/L in WT myocytes (Figure 3A versus Figure 3B) increased CaSpF from 100 to 350 sparks pl⁻¹ s⁻¹. This could be explained by altered RyR gating due to either higher [Ca²⁺]i, or higher SR Ca²⁺ content expected. At 10 nmol/L, the CaSpF in PLB-KO was ≈4 times higher than in the WT. This difference cannot be due to [Ca²⁺]i (which was identical). However, after cAMP addition, WT CaSpF rose to nearly match that of the PLB-KO. This is consistent with the initial 4-fold PLB-KO versus WT difference being due to SR Ca²⁺ content. After PKA phosphorylation of PLB, this difference might be minimized such that SR Ca²⁺ loads are comparable (see below), making CaSpF similar. The failure of CaSpF to change in the PLB-KO myocyte indicates that the cAMP effect on WT CaSpF was entirely attributable to PLB phosphorylation and that RyR effects are not significant.

Figure 3. Ca²⁺ spark frequency versus time (with or without cAMP) in WT, PLB-KO, and PLB-DM. After 7 minutes of control Ca²⁺ spark recording, 10 μmol/L cAMP was applied. Data are mean±SEM of 5 to 17 cells from 3 to 5 mice.
Figure 4 shows grouped data from the 7-minute period before cAMP addition versus the last 5 minutes after cAMP addition, where effects approached steady state. Figure 4A shows the PKA-dependent increase in CaSpF in WT was significant at 10 and 50 nmol/L [Ca\(^{2+}\)]\(_{i}\), (as was the basal PLB-KO versus WT difference). However, cAMP did not affect CaSpF in either PLB-KO or PLB-DM.

Figure 4B shows SR Ca\(^{2+}\) content based on the amplitude of caffeine-induced Ca\(^{2+}\) transients. In WT cells, SR Ca\(^{2+}\) content was increased by cAMP (by 50% and 40% for 10 and 50 nmol/L [Ca\(^{2+}\)]\(_{i}\), respectively). However, there was no cAMP-induced increase in SR Ca\(^{2+}\) in either PLB-KO or PLB-DM. PLB-KO myocytes also had higher basal SR Ca\(^{2+}\) than WT (which explains the higher basal CaSpF in PLB-KO). After cAMP addition, WT myocytes did not quite achieve the SR Ca\(^{2+}\) content of PLB-KO cells, and this might explain the slightly lower mean CaSpF in Figure 4A (WT+cAMP versus PLB-KO).

**Ca\(^{2+}\) Spark Characteristics**

The Table shows fundamental Ca\(^{2+}\) spark characteristics (with or without cAMP). In WT cells, cAMP increased Ca\(^{2+}\) spark amplitude (Δ[Ca\(^{2+}\)]\(_{i}\)), duration (FWHM), and spatial spread (FWHM) in both 10 and 50 nmol/L [Ca\(^{2+}\)]\(_{i}\). The higher SR Ca\(^{2+}\) content in WT after cAMP would increase the amount of Ca\(^{2+}\) released and explains all of these effects. There were no increases in Ca\(^{2+}\) spark characteristics in PLB-KO or PLB-DM after cAMP addition (rather, spark amplitude declined by 7% to 9%). This small reduction is not meaningful, because time-control experiments in PLB-KO showed Ca\(^{2+}\) sparks were 8% smaller in amplitude (P<0.05) and 2% smaller in FWHM (P<0.05) at 10 to 16 minutes versus the first 7 minutes. Thus, PKA-dependent RyR phosphorylation does not affect fundamental Ca\(^{2+}\) spark characteristics.

**Ca\(^{2+}\) Spark Frequency With Maximally Phosphorylated PLB-KO Mouse Myocytes**

D Dephosphorylation of RyR could limit PKA-dependent RyR phosphorylation. To maximize RyR phosphorylation with
cAMP addition, we included phosphatase inhibitors, 10 μmol/L okadaic acid, and 2 mmol/L NaF. Figure 5 shows that Ca\(^{2+}\) spark frequency in PLB-KO myocytes was unaffected by cAMP, even with okadaic acid and NaF.

**Figure 5.** Ca\(^{2+}\) spark frequency in PLB-KO mouse cells (with or without cAMP, with phosphatase inhibitors). CaSpF was not changed by 10 μmol/L cAMP alone or with addition of 10 μmol/L okadaic acid (OA) and 2 mmol/L NaF from 15 minutes, as indicated (data from 6 to 9 PLB-KO myocytes).

**Figure 6.** PKA-dependent phosphorylation of the RyRs in PLB-KO. Four different conditions were used in permeabilized PLB-KO myocytes: (1) minimal phosphorylation (with protein phosphatases PP1 and PKI), (2) control condition (no modulators), (3) cAMP (10 μmol/L), and (4) maximal phosphorylation (with cAMP+10 μmol/L OA+2 mmol/L NaF). A, Measurement of \(^{32}\)P into RyR by autoradiography. The spread below the RyR band may result from degradation. RyR position was verified by Western blot. B, Radioactivity counted by PhosphorImager normalized to control and averaged (n=4).

**Figure 7.** Intact cell resting Ca\(^{2+}\) spark frequency in WT and PLB-KO myocytes (±1 μmol/L isoproterenol). [Ca\(^{2+}\)]\(_o\) was 2 mmol/L and 1 mmol/L for WT and PLB-KO, respectively (to obtain comparable CaSpF). **p<0.01 for control (Ctl) vs isoproterenol (ISO). ISO increased CaSpF in WT (n=5 and 9 for control and ISO condition, respectively), but not in PLB-KO (n=8).

Ca\(^{2+}\) spark measurements (Figure 6). Minimal phosphorylation (lane 1) was 62% of the control basal condition (lane 2). When exposed to 10 μmol/L cAMP (lane 3), RyR phosphorylation was 320% of control, and inclusion of okadaic acid and NaF further enhanced by another 2.4-fold (lane 4). We also increased [cAMP] to 50 μmol/L, with phosphatase inhibitors, but no further phosphorylation was detected. In conclusion, the addition of cAMP as used here greatly increased RyR phosphorylation, presumably approaching an upper limit when phosphatase inhibitors are included.

**Discussion**

In SLO-permeabilized mouse ventricular myocytes, activation of PKA by cAMP causes (1) phosphorylation of the RyR,
(2) increased SR Ca\(^{2+}\) uptake and CaSpF in WT myocytes, (3) no change in either SR Ca\(^{2+}\) load or Ca\(^{2+}\) sparks in the absence of phosphorylatable PLB (PLB-KO and PLB-DM), and (4) no change in CaSpF in PLB-KO, even when RyR phosphorylation is driven to maximally achievable levels. CaSpF was also not altered by PKA activation in intact resting myocytes from PLB-KO mice. We conclude that PKA-dependent RyR phosphorylation does not affect resting SR Ca\(^{2+}\) leak in these myocytes, and that the increased CaSpF seen in WT mice is entirely dependent on PLB phosphorylation and consequent increases in SR Ca\(^{2+}\) content. Moreover, these data provide compelling evidence for the activation of RyR by intra-SR Ca\(^{2+}\) in a relatively intact setting (because [Ca\(^{2+}\)]) was clamped at a constant level).

### Potential Limitations

The present studies were at 23°C versus 37°C. However, all bilayer results suggesting PKA effects on RyR gating were at room temperature. Permeabilization could result in lost accessory proteins or factors required for phosphorylation, but we showed that RyR phosphorylation occurred. We permeabilized with SLO (versus saponin or digitonin) because SLO produces more discrete pores (30 nm in diameter, excluding dextran >148 kDa). Our permeabilized cell studies used 10 to 50 mmol/L [Ca\(^{2+}\)], less than likely diastolic [Ca\(^{2+}\)] in intact cells. However, since purported PKA effects on RyR are to increase its Ca\(^{2+}\) sensitivity, this is where effects should be most apparent. Thus, permeabilization should not be a limitation. CaSpF was higher in permeabilized versus intact cells. Although we do not know why this occurs, it could cause CaSpF to be maximal prior to PKA activation. Low [Ca\(^{2+}\)], was used partly to avoid this limitation, and it is also clear from the WT versus PLB-DM (Figures 3B and 4) that CaSpF could still increase. The similar results in intact versus permeabilized myocytes renders these issues minor.

Conceivably, FKBP 12.6 bound to the RyR is lost during SLO permeabilization, which may be critical in PKA activation of the RyR. That could have raised basal CaSpF versus intact cells and precluded further effects of PKA activation. However, this seems highly unlikely, because FKBP is not dissociated from the RyR even during aggressive homogenization and biochemical purification steps, much more disruptive than SLO exposure. Moreover, the role of FKBP in regulating the cardiac RyR is controversial. We may also not have achieved the level of hyperphosphorylation reported by Marx et al. in HF (4 phosphates per RyR tetramer). However, their PKA effects on RyR channel gating were progressive with increased phosphate incorporation. Thus, if PKA effects are important, we would still have expected to see some alteration in Ca\(^{2+}\) sparks (especially with cAMP plus phosphatase inhibition). This was not the case. The fact that we could drive WT with cAMP to nearly the same SR Ca\(^{2+}\) content (and CaSpF) as in PLB-KO suggests that PKA-dependent phosphorylation was very strongly activated.

It is not possible that PKA caused a significant SR Ca\(^{2+}\) leak that is not detected as Ca\(^{2+}\) sparks in the PLB-KO or PLB-DM. If this were the case, cAMP would have reduced SR Ca\(^{2+}\) content, but that was not observed (Figure 4B). Thus, PKA does not increase SR Ca\(^{2+}\) leak by any significant Ca\(^{2+}\) spark–independent pathway.

### PKA-Dependent Phosphorylation of RyR: Bilayers Versus Intact Myocytes

Valdivia et al. found that PKA decreased basal RyR P_o at 100 nmol/L [Ca\(^{2+}\)]. However, PKA increased peak P_o (to nearly 1.0) during a rapid photolytic increase of local [Ca\(^{2+}\)] and accelerated subsequent P_o decline. Thus, PKA activated cardiac RyR gating dynamically. Marx et al. found that PKA phosphorylated the RyR at Ser-2809, increased P_o at low [Ca\(^{2+}\)], and increased the appearance of subconducting states. CaMKII also phosphorylates cardiac RyR at Ser-2809, but does not seem to produce similar bilayer effects as PKA. Thus, data from single RyRs suggest that RyR phosphorylation can alter gating, but the mechanism is controversial.

If effects at the single RyR channel are physiologically important, effects should be seen in more intact physiological environments. PKA studies in intact ventricular myocytes are complicated by phosphorylation effects on I_{Ca}, SR Ca\(^{2+}\)-ATPase (via PLB phosphorylation), and myofilaments. However, our results on resting CaSpF in intact PLB-KO myocytes, where neither Ca\(^{2+}\) current nor PLB effects should be a factor, strongly support our results in permeabilized myocytes (no CaSpF change with PKA). Although RyR expression is downregulated 25% in PLB-KO mouse, this should not prevent PKA effects on RyRs. In intact WT myocytes, where PLB can be phosphorylated and SR Ca\(^{2+}\) content enhanced, CaSpF increased dramatically as in the permeabilized cells. We conclude that strong PKA-dependent phosphorylation of the RyR does not directly alter resting SR Ca\(^{2+}\) leak via RyR in mouse ventricular myocytes. Rather, the PKA-dependent effects on the resting SR are mainly due to PLB phosphorylation.

During cardiac E-C coupling, PKA effects are more complicated to assess because PKA activation increases both I_{Ca} and SR Ca\(^{2+}\) content. In this case, increased Ca\(^{2+}\) transient amplitude provides no information about intrinsic RyR alterations. The ideal way to assess this is to control I_{Ca} and SR Ca\(^{2+}\) content so they are the same before and after PKA activation. Then the SR Ca\(^{2+}\) release for a given I_{Ca} and SR Ca\(^{2+}\) can be compared. Li et al. found that Ca\(^{2+}\)-dependent CaMKII activation increases the fraction of SR Ca\(^{2+}\) released for a given I_{Ca} and SR Ca\(^{2+}\) load. For PKA activation, preliminary data of this sort do not indicate large changes in E-C coupling. Two recent studies that partially fulfill this design reported that PKA either increases or decreases E-C coupling gain. It was also argued that altered RyR gating (alone) during systole can only produce transient changes in steady-state twitch Ca\(^{2+}\) transients, although preferential enhancement of diastolic SR Ca\(^{2+}\) leak can unload the SR and contribute to negative inotropy. Thus, although the present results indicate that PKA does not alter resting SR Ca\(^{2+}\) leak, the E-C coupling effects are complex and unresolved. Indeed, it would be valuable to explore whether the PKA-induced enhancement of RyR opening in response to rapid local [Ca\(^{2+}\)], changes seen in bilayers is evident during E-C coupling in more intact cellular systems.
Luminal Ca\(^{2+}\) Effects on RyR Gating

Luminal (intra-SR) [Ca\(^{2+}\)] can importantly affect cardiac RyR gating. Indeed, high luminal [Ca\(^{2+}\)] greatly increases Ca\(^{2+}\) sensitivity of RyR activation in bilayer recording.\(^{39-42}\) This is also seen for Ca\(^{2+}\) sparks\(^{43,44}\) (but see also Reference 26); however, in intact cells, it is difficult to unequivocally control cytosolic free [Ca\(^{2+}\)]. The present data and a related skinned myocyte Ca\(^{2+}\) spark study\(^{45}\) have shown that at constant [Ca\(^{2+}\)], increasing SR Ca\(^{2+}\) content can dramatically increase CaSpF (and that lower SR Ca\(^{2+}\) content decreases CaSpF). Luminal Ca\(^{2+}\) sensitivity also extends to E-C coupling, where fractional SR Ca\(^{2+}\) release is nearly zero at about half-maximal SR Ca\(^{2+}\) load, but increases very steeply as load increases.\(^{46,47}\)

Overall, we conclude that PKA-dependent increase in CaSpF and spark amplitude in mouse ventricular myocytes is attributable to PLB phosphorylation and consequent enhancement of SR Ca\(^{2+}\) load. PKA does not appear to appreciably affect resting RyR function in these myocytes. It will be a challenge to reconcile these results with those indicating PKA-dependent modulation of RyR gating in HF.\(^{12}\) Additionally, we provide compelling evidence that elevated intra-SR [Ca\(^{2+}\)] causes increased CaSpF, independent of [Ca\(^{2+}\)], which was clamped.

Acknowledgments

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