Ca^{2+}/Calmodulin-Dependent Protein Kinase II
Phosphorylation of Ryanodine Receptor Does Affect
Calcium Sparks in Mouse Ventricular Myocytes

Tao Guo, Tong Zhang, Ruben Mestril, Donald M. Bers

Abstract—Previous studies in transgenic mice and with isolated ryanodine receptors (RyR) have indicated that Ca^{2+}-calmodulin-dependent protein kinase II (CaMKII) can phosphorylate RyR and activate local diastolic sarcoplasmic reticulum (SR) Ca^{2+} release events (Ca^{2+} sparks) and RyR channel opening. Here we use relatively controlled physiological conditions in saponin-permeabilized wild type (WT) and phospholamban knockout (PLB-KO) mouse ventricular myocytes to test whether exogenous preactivated CaMKII or endogenous CaMKII can enhance resting Ca^{2+} sparks. PLB-KO mice were used to preclude ancillary effects of CaMKII mediated by phospholamban phosphorylation. In both WT and PLB-KO myocytes, Ca^{2+} spark frequency was increased by both preactivated exogenous CaMKII and endogenous CaMKII. This effect was abolished by CaMKII inhibitor peptides. In contrast, protein kinase A catalytic subunit also enhanced Ca^{2+} spark frequency in WT, but had no effect in PLB-KO. Both endogenous and exogenous CaMKII increased SR Ca^{2+} content in WT (presumably via PLB phosphorylation), but not in PLB-KO. Exogenous calmodulin decreased Ca^{2+} spark frequency in both WT and PLB-KO (K_{0.5} \approx 100 \text{ nmol/L}). Endogenous CaMKII (at 500 nmol/L [Ca^{2+}]) phosphorylated RyR as completely in <4 minutes as the maximum achieved by preactivated exogenous CaMKII. After CaMKII activation Ca^{2+} sparks were longer in duration, and more frequent propagating SR Ca^{2+} release events were observed. We conclude that CaMKII-dependent phosphorylation of RyR by endogenous associated CaMKII (but not PKA-dependent phosphorylation) increases resting SR Ca^{2+} release or leak. Moreover, this may explain the enhanced SR diastolic Ca^{2+} leak and certain triggered arrhythmias seen in heart failure. (Circ Res. 2006;99:398-406.)

Key Words: cardiac myocytes ■ sarcoplasmic reticulum ■ Ca spark ■ CaMKII

Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) are central in excitation contraction coupling (ECC) both as the sarcoplasmic reticulum (SR) Ca^{2+} release channel and as a scaffolding protein that localizes numerous regulatory proteins to the junctional complex.\textsuperscript{1} Ca^{2+} sparks reflect the synchronous activation of a cluster of \(\approx 6\) to \(20\) RyR at a single junction, producing both the diastolic SR Ca^{2+} leak and the temporally synchronized SR Ca^{2+} release during ECC.\textsuperscript{1,3} Their activity can be modulated by many factors including divalent cations, adenine nucleotides, calmodulin (CaM), caffeine, oxidation, and phosphorylation.\textsuperscript{1,2}

Protein kinase A (PKA)-dependent RyR phosphorylation has been reported to increase RyR open probability at the single channel level.\textsuperscript{4-6} It was further suggested that hyperphosphorylation of RyR at Ser\textsuperscript{209} by PKA in heart failure (HF) caused dissociation of FK-506 binding protein (FKBP12.6) from the RyR, resulting in enhanced diastolic Ca^{2+} leak, reduced SR Ca^{2+} content, and contractile dysfunction.\textsuperscript{7} However, Li et al\textsuperscript{8} found that cAMP-induced RyR phosphorylation had no effect on resting RyR-mediated SR Ca^{2+} leak (assessed via Ca^{2+} sparks) in phospholamban (PLB) knockout mouse myocytes (PLB-KO). It remains controversial whether PKA-dependent RyR phosphorylation does occur in HF and whether it causes dissociation of FKBP12.6 from RyR or mediates enhanced SR Ca^{2+} leak.\textsuperscript{8-10}

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) has been reported to phosphorylate or regulate L-type Ca^{2+} current (I_{Ca}), PLB, and RyR.\textsuperscript{11-16} At the single-channel recording level, reports suggest that CaMKII can either enhance\textsuperscript{5,16,17} or depress RyR activity.\textsuperscript{18} However, in voltage clamped intact myocytes, Li et al\textsuperscript{19} reported that endogenous CaMKII increased SR Ca^{2+} release for a given SR Ca^{2+} content and I_{Ca} trigger. In addition, protein phosphatases (PP1 and PP2A) can reduce SR Ca^{2+} release channel activity for a given SR load and I_{Ca},\textsuperscript{20} but can also enhance Ca^{2+} spark frequency.\textsuperscript{21} Moreover, in transgenic mice overexpressing CaMKII\textsubscript{c}, fractional SR Ca^{2+} release was enhanced and resting spontaneous SR Ca^{2+} spark frequency was dramatically increased, despite lower SR Ca^{2+} load and lower diastolic [Ca^{2+}].\textsuperscript{22} Moreover, CaMKII is associated with RyR\textsuperscript{22} and is upregulated in heart failure,\textsuperscript{22} where SR Ca^{2+} leak may contribute to systolic dysfunction and arrhythmogenesis.\textsuperscript{22}
The goal here was to test the effects of CaMKII on resting SR Ca\(^{2+}\) release (as Ca\(^{2+}\) sparks) in saponin-permeabilized mouse ventricular myocytes (wild type [WT] and PLB-KO). Activation of endogenous CaMKII or application of preactivated exogenous CaMKII (autophosphorylated) resulted in RyR phosphorylation and enhanced resting Ca\(^{2+}\) spark frequency (CaSpF), independent of SR Ca\(^{2+}\) load. CaM also depressed RyR activity with a half-maximal effect at 100 nmol/L CaM.

**Methods**

**Cardiac Myocyte Isolation**

Mouse ventricular myocytes were enzymatically isolated as previously described (see the online data supplement, available at http://circres.ahajournals.org),\(^8\) and PLB-KO mice were provided by Dr E.G. Kranias (University of Cincinnati, OH). Procedures were performed according to Guiding Principles in the Care and Use of the Animals approved by the Council of the American Physiological Society.

**Ca\(^{2+}\) Sparks in Permeabilized Myocytes**

Myocytes were permeabilized with saponin (50 μg/mL) for 30 seconds (see the online data supplement for details) and placed in internal solution (in mmol/L): EGTA 1, HEPES 10, K-aspartate 120, ATP 5; free MgCl\(_2\) 1; reduced glutathione 10, free [Ca\(^{2+}\)] 50 nmol/L for WT and 10 or 25 mmol/L for PLB-KO (see the online supplement), creatine phosphokinase 5 U/mL, phosphocreatine 10, dextran (relative molecular mass: 40 000) 4%, K\(_4\)Fluo-3 0.05, pH 7.2. PKA inhibitory peptide PKI (15 μmol/L; Calbiochem no. 116805) was in all bath solutions (except when exogenous PKA was added). After baseline Ca\(^{2+}\) sparks were recorded, myocytes were exposed to solution designed to phosphorlyate RyR (see below). After this period, solution was replaced by the original internal solution containing 10 μmol/L okadaic acid (OA) to prevent dephosphorylation in subsequent Ca\(^{2+}\) spark measurements. Control experiments showed that OA by itself (5 minutes) caused no change in CaSpF in 19 of 20 myocytes. Ca\(^{2+}\) sparks were recorded as previously described.\(^8\) SR Ca\(^{2+}\) load was evaluated by Ca\(^{2+}\) transient amplitude on caffeine application. CaSpF is expressed as sparks per picoliter per second (p1\(^{-1}\)s\(^{-1}\)).

**Activation of Endogenous CaMKII**

To activate endogenous CaMKII in permeabilized ventricular myocytes [Ca\(^{2+}\)]\(_{\text{cyt}}\) was elevated for 1 minute to 500 mmol/L with 1.2 μmol/L exogenous CaM. Phosphatase inhibitor OA (2 μmol/L) was included to prevent dephosphorylation of CaMKII and its targets, and 15 μmol/L PKI was included.

**Preactivation of Exogenous CaMKII**

We also used preactivated exogenous CaMKII. CaMKIIa (12 mg/mL; gift from Dr J.H. Brown, University of California, San Diego) was preactivated (autophosphorylated) by incubation with (in μmol/L): ATP 100, CaM 2.4, and CaCl\(_2\) 200 for 10 minutes at 37°C. In some experiments, CaMKII inhibitor AIP (1 μmol/L) was included as a negative control. Preactivated CaMKII was then rapidly diluted into final superfusate with free [Ca\(^{2+}\)] restored to 50 nmol/L (with 6 μg/mL CaMKII, 1.2 μmol/L CaM, 10 μmol/L OA, and 15 μmol/L PKI) and exposed to permeabilized myocytes.

**Phosphorylation and CaMKII**

**Phosphorylation of RyR**

RyR was immunoprecipitated from homogenate or cell lysate with antibody (MA3–916, Affinity BioReagents) in 0.5 mL of 62.5 mmol/L Tris–HCl buffer (pH 7.5), 0.9% NaCl, 0.5 mmol/L NaF, 1% Triton-X, and protease inhibitors for 2 hours at 4°C (see the online supplement for details). CaMKII phosphorylation of immunoprecipitated RyR was initiated by adding autophosphorylated CaMKII and yP\(^{2+}\)-ATP (final specific activity 300 μCi/μmol). Reactions were terminated with stop solution (10% sodium dodecyl sulfate, 300 mmol/L EGTA, and 0.25 mmol/L DTT) and size-fractionated on 5% SDS-PAGE, and RyR radioactivity was quantified using Unscan-it software. RyR protein was determined by immunoblotting. FKBP12.6 was measured\(^8\) using anti-FKBP12 (supplied by Dr A.R. Marks, Columbia University, New York; see the online supplement).

**Data Analysis**

Ca\(^{2+}\) sparks were analyzed as previously described (see the online supplement).\(^8\) Ca\(^{2+}\) spark amplitudes were normalized to fluorescence baseline (F\(_0\)) as F/F\(_0\), duration was full-duration half-maximum (FDHM), and width was full-width half-maximum (FWHM). Results are expressed as mean±SEM. Significance (P<0.05) was determined using Student t test.

**Results**

**Exogenous CaMKII on Ca\(^{2+}\) Sparks and SR Ca\(^{2+}\) Content in WT mice**

Figure 1A shows representative line scan images from a WT myocyte before, during application of preactivated CaMKIIa with CaM, and following washout of CaM/CaMKIIa (with dephosphorylation inhibited by OA). Figure 1B shows mean data, where CaSpF did not increase during CaM/CaMKII exposure for 5 minutes (205±20 versus 199±39 sparks pl\(^{-1}\)s\(^{-1}\)), but 5 minutes after washout of CaM/CaMKII CaSpF greatly increased (by 108%, n=10) versus control.

The dramatic increase in CaSpF during CaM/CaMKII washout is consistent with CaMKII-dependent activation of RyR. However, the failure to see increased CaSpF during CaM/CaMKII exposure could be attributable to an acute inhibitory effect of CaM (see CaM Alone Inhibits Ca\(^{2+}\) Spark Frequency).

CaMKII can phosphorylate PLB to cause increased SR Ca\(^{2+}\) uptake and content, and CaSpF depends strongly on SR Ca\(^{2+}\) content.\(^1,8\) Figure 1C shows that after exposure to CaM/CaMKII there was a small (14%), but significant increase in SR Ca\(^{2+}\) content (assessed by caffeine-induced Ca\(^{2+}\) transients). Given the steep leak versus SR Ca\(^{2+}\) content relationship,\(^1,8\) we cannot rule out the possibility that part of the 108% increase in CaSpF after CaMKII exposure was secondary to the 14% increase in SR Ca\(^{2+}\) content.

**Exogenous CaMKII on Ca\(^{2+}\) Sparks and SR Ca\(^{2+}\) Content in PLB-KO Mice**

To eliminate the potential complicating effect of CaMKII on PLB we repeated the same protocol in PLB-KO mice (Figure 2). In PLB-KO myocytes, CaM/CaMKIIa addition raised the mean CaSpF by 28%, but not significantly (P=0.06). After washout, CaSpF increased significantly by 60% (P<0.0001, n=9; Figure 2B). In PLB-KO myocytes, SR Ca\(^{2+}\) content was not increased by exposure to activated CaMKII (Figure 2C).

Thus, the increased CaSpF cannot be secondary to increased SR Ca\(^{2+}\) content, but could be caused by CaMKII-dependent RyR modulation. As a control, these experiments were also done with CaMKII inhibitory peptide AIP in the CaMKII preactivation cocktail. Figure 2D shows that AIP prevented the increase in CaSpF. Thus CaMKII activity is required for the observed increase in CaSpF.
There were small, but significant changes in Ca$^{2+}$/H$^{110}$ spark characteristics (Table). For example, there was larger spark duration and spatial spread after exposure to activated CaMKII (in both WT and PLB-KO). These observations would be consistent with CaMKII causing minor increases in the duration of SR Ca$^{2+}$ release during Ca$^{2+}$ sparks. The small increase in Ca$^{2+}$ spark amplitude in WT cells during CaM/CaMKII exposure might be secondary to the increase in SR Ca$^{2+}$ content caused by the combination of SR Ca-ATPase stimulation (via PLB phosphorylation) and inhibition of SR Ca$^{2+}$ leak (via CaM, see CaM Alone Inhibits Ca$^{2+}$ Spark Frequency).

**Figure 1.** CaMKII increases Ca$^{2+}$ spark frequency in WT mouse ventricular myocytes. A, Longitudinal line scan images of Ca$^{2+}$ sparks in a permeabilized WT myocyte under control conditions (left), 3 minutes after exposure to preactivated CaMKII (middle) and 3 minutes after washout of CaM and CaMKII (all at 50 nmol/L [Ca$^{2+}$]). B, Pooled data for CaSpF (left) and SR Ca$^{2+}$ content assessed by the ΔCa$^{2+}$ on rapid caffeine application (n=5 myocytes; *P<0.05).

**Figure 2.** CaMKII increases Ca$^{2+}$ spark frequency in PLB-KO myocytes. A, Ca$^{2+}$ sparks in a permeabilized PLB-KO myocyte under control conditions (left), 3 minutes after exposure to preactivated CaMKII (middle), and 3 minutes after washout (all at 25 nmol/L [Ca$^{2+}$]). Pooled data for (B) CaSpF and (C) SR Ca$^{2+}$ content assessed by caffeine-induced Δ[Ca$^{2+}$] (right; n=6 PLB-KO myocytes; *P<0.05). D, Pooled results, n=5 experiments where 1 μmol/L AIP was included throughout.
CaM Alone Inhibits Ca^{2+} Spark Frequency

In both WT and PLB-KO mice, we often saw an initial acute inhibition of CaSpF, which we hypothesized was caused by CaM-dependent RyR inhibition as observed in bilayer and SR vesicle studies.\textsuperscript{23,24} To test whether CaM had direct effects on RyR (versus via CaMKII), we applied different CaM concentrations to myocytes under conditions where CaMKII should not be activated: (1) in WT myocytes at 50 nmol/L [Ca^{2+}], without CaMKII inhibitor AIP (this low [Ca^{2+}] should not activate CaMKII; Figure 3A) and (2) in PLB-KO myocytes at 25 nmol/L [Ca^{2+}], with AIP (to inhibit any endogenous CaMKII; Figure 3B). The right panels of Figure 3 show that CaM decreased CaSpF in a dose-dependent manner with half-inhibition at \( \approx 100 \) nmol/L CaM (K\textsubscript{0.5}) in both WT and PLB-KO myocytes. Furthermore, the inhibitory effect was partially reversed by CaM washout (Figure 3A and 3B). CaM did not alter Ca^{2+} spark properties (not shown).

Exogenous PKA and CaSpF

The approach used here previously showed that cAMP-dependent RyR phosphorylation could increase CaSpF in WT, but not in PLB-KO mice (or where only nonphosphorylatable PLB was expressed).\textsuperscript{8} Here, we further test whether exogenous PKA catalytic subunit alters CaSpF, as we see for preactivated CaMKII. Figure 4B shows that exogenous PKA increased CaSpF in WT, but not in PLB-KO. These results with active PKA agree with the earlier results with cAMP (which depended on endogenous PKA).\textsuperscript{8} However, the PKA results contrast dramatically with the present results with activated CaMKII.
Because the PKA effect on RyR may depend on dissociation of FKBP12.6, we also measured FKBP12/12.6 expression and RyR association. There is no change in total FKBP12 expression in PLB-KO versus WT hearts (Figure 4C). Nor was there any difference in FKBP12.6, which coimmunoprecipitates with RyR in control conditions or after treatment with PKA or PKI (Figure 4D). We also did not find any appreciable loss of RyR-associated FKBP12.6 on myocyte permeabilization as used here (not shown). Thus, we did not detect FKBP12.6 dissociation under conditions where in WT myocytes there was enhanced CaSpF (presumably because of PLB phosphorylation, enhanced SR Ca-ATPase activity, and SR Ca content).\(^8\)

**Endogenous CaMKII on Ca\(^{2+}\) Sparks in Permeabilized Myocytes**

Since endogenous CaMKII is known to associate with the RyR,\(^{17,25-27}\) we sought to test whether endogenous CaMKII can activate RyR in myocytes as seen above for exogenous CaMKII. Figure 5A shows line scans during a 5-minute control period, as \([\text{Ca}^{2+}]_i\) was elevated to 500 nmol/L (with 1.2 Ìmol/L CaM, 15 Ìmol/L PKI, and 2 Ìmol/L OA added to activate endogenous CaMKII) and on washout (where CaM was removed and \([\text{Ca}^{2+}]_i\) restored to the initial level). The same protocol was performed with the specific CaMKII inhibitor peptide (AIP 1 Ìmol/L) included during and after \([\text{Ca}^{2+}]_i\) and CaM elevation. AIP prevented the increase in CaSpF in both WT and PLB-KO (Figure 5B and 5C). SR Ca\(^{2+}\) content was significantly increased in WT myocytes, but did not change significantly in PLB-KO. This demonstrates that the increased CaSpF was not caused by increased SR load in the PLB-KO. These results are consistent with exogenous CaMKII effects on both PLB and RyR.

In WT myocytes PLB-dependent stimulation of SR Ca-ATPase must be slightly stronger than the enhanced SR Ca\(^{2+}\) leak, resulting in higher SR Ca\(^{2+}\) content. However, in PLB-KO, the enhanced SR Ca\(^{2+}\) leak should lower SR Ca\(^{2+}\) content, but did not. This may be because the SR Ca\(^{2+}\) leak does not greatly depress load (see Figure II in the online supplement).

Ca\(^{2+}\) spark amplitude increased in WT myocytes on endogenous CaMKII activation (Table), possibly because of enhanced SR Ca\(^{2+}\) content and spark duration (FDHM). In PLB-KO, spark amplitude also increased, possibly because of prolonged release duration. Figure 6A shows histograms of Ca\(^{2+}\) spark durations (plus activation of endogenous CaMKII) in PLB-KO myocytes. For control, 90% of Ca\(^{2+}\) sparks were 10 to 40 ms in FDHM, whereas after CaMKII this value was only 77%. There were essentially no Ca\(^{2+}\) sparks with FDHM >70 ms in control (0.3% of events), but CaMKII increased this to 4.7% of the already more frequent events. Integrating the individual Ca\(^{2+}\) spark events (from longest to shortest
duration, Figure 6B) shows that half the Ca$^{2+}$ sparks are longer than 32 ms in CaMKII versus 26 ms in control. Figure 6C shows that CaMKII activation resulted in occasional macrosparks (that exceed the spatial spread of normal Ca$^{2+}$ sparks; 0.11±0.03/s) and miniwaves (propagating Ca-induced Ca-release; 0.021±0.011/s), but these events were virtually absent in control (0.008±0.0003/s macrosparks and 0 miniwaves). The much higher frequency of long Ca$^{2+}$ sparks and propagating SR Ca$^{2+}$ release events may reflect a higher propensity for initiation of delayed afterdepolarizations (DADs) and consequent triggered arrhythmias on CaMKII-dependent RyR phosphorylation.

**Phosphorylation of RyR and PLB in WT Mouse Ventricular Myocytes**

Finally, we assessed CaMKII-dependent RyR phosphorylation after treating ventricular myocytes as in the Ca$^{2+}$ spark measurements. Here we used RyR back-phosphorylation after myocyte incubation by subsequent treatment with CaMKII (and 32P-ATP, high [Ca$^{2+}$], and [CaM]). In this case the amount of 32P incorporated is highest when the least RyR phosphorylation already occurred during the primary incubation (ie, basal and AIP+alkaline phosphatase–treated samples in Figure 7A, lane 2 and 1). Thus, the reciprocal of back-phosphorylation is an index of prior phosphorylation (Figure 7B). Ca/CaM-treated myocytes

Figure 5. Endogenous CaMKII activation increases Ca spark frequency. A, Representative line scans in PLB-KO at 10 nmol/L [Ca$^{2+}$], on addition of 1.2 μmol/L CaM with 500 nmol/L [Ca$^{2+}$], and at 5 and 8 minutes after washout of CaM and return of [Ca$^{2+}$] to 50 nmol/L. B, Pooled data from this protocol in 6 WT myocytes ([Ca$^{2+}$]i=50 nmol/L) and (C) 9 PLB-KO myocytes (Bar indicates time of Ca/CaM elevation). D, SR Ca$^{2+}$ content (caffeine-induced [Ca$^{2+}$]i) in WT (n=5, P<0.05) and PLB-KO in both control (Ctl) and after CaMKII activation/washout (w/o; n=5).

Figure 6. Ca$^{2+}$ sparks duration after endogenous CaMKII activation in PLB-KO. A, Histograms of Ca spark duration (FDHM), normalized to total number of events in each case (703 for Control, 2408 for CaMKII). B, Integration of events from longest to shortest duration. Half of events were longer than 32 ms in CaMKII and 26 ms in control. C, Frequency of macrosparks (FWHM >5 μm) and miniwaves (example in inset), where the latter are counted as 2 macrosparks. Note that fully propagating waves are greatly limited by the 1 mmol/L EGTA in permeabilized fiber solution.
had nearly maximal phosphorylation levels, similar to when cells were exposed to exogenous activated CaMKIIα. The time course of in vivo RyR phosphorylation is consistent with that of CaSpF (Figure 3B). Myocytes treated with AIP and alkaline phosphatase had the least PLB phosphorylation (lane 2), suggesting the basal RyR phosphorylation by CaMKII is very low.

CaMKII also phosphorylates PLB at Thr-17, and this is a control for CaMKII target phosphorylation. Figure 7A and 7C shows that basal PLB phosphorylation was too low to detect. After treatment with Ca/CaM (even after restoration of leak via Ca2⁺ leak, global Ca2⁺ sparks, and (3) CaM decreases CaSpF (although SR Ca2⁺ content was not measured in the same protocols). Currie et al. used permeabilized rabbit ventricular myocytes and found that the CaMKII inhibitor AIP inhibited CaSpF and ryanodine binding (an indicator of RyR activation). Thus, our results and most others indicate that endogenous CaMKII activity can phosphorylate RyR and enhance RyR opening, both at rest (as SR Ca2⁺ leak or Ca2⁺ sparks), and during E-C coupling.

CaM and Resting SR Ca2⁺ Release

Our control CaM studies indicate that CaM depresses RyR activity in the cellular setting at diastolic [Ca2⁺], with a K₀.₅ for CaM ≈ 100 nmol/L. This is consistent with extensive earlier work on CaM binding to and inhibition of RyR gating in isolated RyR in bilayers or vesicles, with respect to both Kᵢ and binding kinetics (ie, CaSpF changed over tens of seconds). Notably, this K₀.₅ is comparable to the free [CaM] in ventricular myocytes at diastolic [Ca2⁺], such that gradual changes in RyR regulation could occur as free [CaM] changes (although significant changes of RyR-CaM binding during a single heartbeat are unlikely).

At high free [CaM] (≥ 2 μmol/L), inhibition of CaSpF began to reverse in an AIP-sensitive manner. This indicates that some local CaMKII activation may occur even at diastolic [Ca2⁺], when [CaM] is high. This emphasizes the importance of quantitative studies of CaM effects in a cellular environment.

Previous Results Concerning CaMKII and Resting Ca2⁺ Release

Most earlier studies of cardiac RyR gating in lipid bilayers have shown that CaMKII enhances RyR open probability, but not all results agree. A recent surprising result in bilayers was that phosphatase treatment also increased RyR activity in ≈ 50% of channels studied. Discrepancies among bilayer studies are unresolved.

Similarly, most work in intact ventricular myocytes suggests that CaMKII enhances cardiac RyR activation. Transgenic mice overexpressing CaMKIIΔC show increased CaMKII associated with RyR, enhanced RyR phosphorylation, and increased fractional SR Ca2⁺ release and resting CaSpF (despite lower SR Ca2⁺ content and diastolic [Ca2⁺]). Acute CaMKIIΔC overexpression in rabbit ventricular myocytes (via adenovirus) increased fractional SR Ca2⁺ release and CaSpF (normalized for SR Ca2⁺ content). When SR Ca2⁺ content and Lᵢᵢ trigger were controlled and matched, activation of endogenous CaMKII greatly enhanced fractional SR Ca2⁺ release, whereas CaMKII inhibition (by AC3-I) enhanced SR Ca2⁺ release (although SR Ca2⁺ content was not measured in the same protocols). Currie et al. used permeabilized rabbit ventricular myocytes and found that the CaMKII inhibitor AIP inhibited CaSpF and ryanodine binding (an indicator of RyR activation). Thus, our results and most others indicate that endogenous CaMKII activity can phosphorylate RyR and enhance RyR opening, both at rest (as SR Ca2⁺ leak or Ca2⁺ sparks), and during E-C coupling.

Discussion

We demonstrated in a controlled mouse PLB-KO myocyte setting that: (1) CaMKII-dependent RyR phosphorylation significantly increases CaSpF and duration, independent of SR Ca2⁺ content (using both exogenous and endogenous CaMKII), (2) exogenous PKA catalytic subunit does not activate SR Ca2⁺ leak via Ca2⁺ sparks, and (3) CaM decreases CaSpF with Kᵢ ≈ 100 nmol/L [Ca2⁺].

Most previous work has removed the RyR from its native cellular environment (SR vesicles or single-channel bilayer recordings) or used intact myocytes where [Ca2⁺], SR Ca2⁺ content, and CaMKII activation are difficult to control. Our permeabilized myocytes bridge these, in that [Ca2⁺], SR Ca2⁺ content, and CaMKII activity are relatively controlled and the local RyR environment is minimally perturbed. In addition, PLB-KO myocytes prevent the major complicating effect of both CaMKII and PKA on the SR Ca-ATPase. With PLB present, activation of PKA and CaMKII can increase SR Ca2⁺ content, which itself alters CaSpF, SR Ca2⁺ leak, global Ca2⁺ transients, and Ca2⁺ spark amplitude.
WT mouse myocytes had similar behavior to PLB-KO here (see the online supplement). However, conclusions for WT are complicated by CaMKII phosphorylation of PLB at Thr-17. CaMKII stimulation of both SR Ca-ATPase and RyR-mediated SR Ca\(^{2+}\) leak would affect SR Ca\(^{2+}\) content oppositely. In WT, the SR Ca\(^{2+}\)-ATPase stimulation predominates with respect to SR Ca\(^{2+}\) content (which was slightly increased). However, both effects would tend to enhance SR Ca\(^{2+}\) leak and CaSpF. This emphasizes the value of PLB-KO mice in isolating RyR effects mechanistically. It also suggests that these combined actions in normal (or failing) myocytes could be more arrhythmogenic than either alone.

**Does RyR Become Phosphorylated by CaMKII Under Physiological Conditions?**

Wehrens et al\(^{17}\) showed that RyR phosphorylation at Ser2815 by CaMKII increases at higher heart rate, and the same was found in isolated myocytes.\(^{33}\) Increasing heart rate also increases CaMKII increases at higher heart rate, and the same was found in isolated myocytes.\(^{33}\) However, both effects would tend to enhance SR Ca\(^{2+}\) leak and CaSpF. This emphasizes the value of PLB-KO mice in isolating RyR effects mechanistically. It also suggests that these combined actions in normal (or failing) myocytes could be more arrhythmogenic than either alone.

**Ca\(^{2+}\) Leak in Heart Failure: Pathological Implications**

CaMKII is upregulated in HF in humans\(^{36}\) and animals.\(^{27,37}\) In an arrhythmogenic rabbit HF model, more CaMKII (and less phosphatase 1 and 2A) is associated with RyR, CaMKII is more activated (autophosphorylated), and RyR is more highly phosphorylated.\(^{27}\) In addition, there is increased diastolic SR Ca\(^{2+}\) leak that can be inhibited by CaMKII blockers,\(^{27,38}\) as was seen on CaMKII overexpression.\(^{32,39}\) There was also a decrease in RyR-bound CaM in this HF model,\(^{37}\) which could further enhance SR Ca\(^{2+}\) leak. Thus, in HF there is likely to be CaMKII-dependent enhancement of diastolic SR Ca\(^{2+}\) leak via RyR, Ca\(^{2+}\) sparks, macrosparks, and Ca\(^{2+}\) waves. These diastolic SR Ca\(^{2+}\) release events can contribute to reduced SR Ca\(^{2+}\) content.\(^{38}\) However, they are also believed to underlie transient inward currents and delayed afterdepolarizations, which can initiate ventricular tachycardia, and the incidence of these events is increased in HF.

In conclusion, we demonstrated that activation of CaMKII (endogenous or exogenous) and reduced [CaM] greatly enhance resting SR Ca\(^{2+}\) release events (Ca\(^{2+}\) sparks and waves) in permeabilized ventricular myocytes under relatively controlled physiological conditions. Thus, CaMKII-dependent effects may normally serve as a positive influence on Ca\(^{2+}\) transients at higher heart rate (eg, by enhancing fractional release during E-C coupling in association with enhanced SR Ca\(^{2+}\)) content. However, when phosphorylation is perturbed in pathophysiological states such as HF, inappropriately high RyR phosphorylation by CaMKII may contribute to reduced SR Ca\(^{2+}\) content and arrhythmogenesis.

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Disclosures
None.

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Ca/calmodulin-dependent protein kinase II phosphorylation of Ryanodine Receptor Does Affect Calcium Sparks in Mouse Ventricular Myocytes

by
Tao Guo, Tong Zhang, Ruben Mestril, Donald M. Bers

METHODS (expanded)

Cardiac Myocyte Isolation
Single mouse ventricular myocytes were enzymatically isolated as previously described. and PLB-KO mice were provided by Dr. E.G. Kranias (University of Cincinnati, OH). All procedures were performed according to the Guiding Principles in the Care and Use of the Animals approved by the Council of the American Physiological Society. Briefly, after anesthesia (isoflurane, 5%), hearts were excised and perfused (5 min, 37°C) with the minimal essential medium (MEM, GIBCO Life Technologies) gassed with 95% O2/5% CO2, before inclusion of collagenase B (0.5 mg/ml, Boehringer Mannheim) and protease (0.02 mg/ml, Sigma). Triturates were incubated (10 min, 37°C) in the same enzyme solution, washed, and kept in 100 µmol/L Ca2+ MEM solution.

Ca2+ Sparks in Permeabilized Cells using Confocal Microscopy
Ventricular myocytes were superfused with relaxing solution containing (in mmol/L) EGTA 0.1, ATP 5, HEPES 10, potassium aspartate 150, MgCl2 0.25, and reduce-glutathione 10, at 23°C. The sarcolemma was permeabilized with saponin (50 µg/ml) for 30s. After permeabilization, myocytes were placed in internal solution composed of (mmol/L): EGTA 1; HEPES 10; K-aspartate 120; ATP 5; free MgCl2 1; reduced glutathione 10; free [Ca2+] 50 nmol/L for wild type (WT), and 25 nmol/L for PLB-KO (calculated using MaxChelator), creatine phosphokinase 5 U/ml; phosphocreatine 10; dextran (Mr: 40,000) 4%; fluo-3 potassium salt 0.05; pH 7.2. To prevent possible basal PKA activity, we included the PKA inhibitory peptide PKI (15 µmol/L from Calbiochem, catalog #116805) in all the bath solutions (except in experiments when exogenous PKA was added). Each cardiomyocyte was first superfused with 50 nM [Ca2+], (or 25 nM for PLB-KO mice) internal solution and baseline Ca2+ sparks were recorded. Then, the permeabilized myocyte was exposed to solution designed to phosphorylate the RyR (either by endogenous CaMKII or exogenous pre-activated CaMKII or PKA catalytic subunit; see below). After this period, the phosphorylating solution was washed out and replaced by the original internal solution containing 10 µM okadaic acid (OA) to prevent dephosphorylation while Ca2+ sparks were again measured. Preliminary control experiments were done in 20 myocytes to test whether OA alone changes the Ca2+ spark frequency. After incubation with OA for 5 min, 19 showed no change in CaSpF, 1 showed increase of CaSpF. Ca2+ sparks were recorded by a laser scanning confocal microscope (Radiance 2000 MP, Bio-Rad, UK) as previously described. SR Ca2+ load was evaluated by Ca2+ transient amplitude upon caffeine application. The amplitude of caffeine-induced Ca transients cannot be simply compared between different [Ca2+] solution, because Ca2+ buffering differs (EGTA saturation level) and may compete with diffusion differently.

Activation of Endogenous CaMKII
To activate endogenous CaMKII in permeabilized ventricular myocytes [Ca2+], was elevated for 1 min to 500 nM with 1.2 µM exogenous calmodulin. Phosphatase inhibitor okadaic Acid (OA) 2 µM was included to prevent dephosphorylation of CaMKII and its targets and 15 µM PKI was included.

Pre-activation of Exogenous CaMKII
An alternative approach was to expose the permeabilized myocyte to exogenous CaMKII that was pre-activated by autophosphorylation (which allows CaMKII to remain active, even after [Ca2+] declines) CaMKIIα 12 mg/ml (kind gift from Dr. J.H. Brown, University of California, San Diego) was pre-activated by incubation with (in µmol/L) 100 ATP, 2.4 calmodulin and 200 CaCl2 for 10 min at 37°C.
In some experiments, CaMKII inhibitor AIP (1 µM) was included for a negative control. This pre-activated CaMKII with Ca/CaM was then rapidly diluted into final superfusate with free [Ca\(^{2+}\)] restored to 50 nM (with 6 µg/ml CaMKII, 1.2 µM CaM, 10 µM OA and 15 µM PKI) and then exposed to the permeabilized myocyte.

**Immunoprecipitation and CaMKII Phosphorylation of RyR2**

RyR2 channels were immunoprecipitated from 500 µg homogenate or cell lysate with antibody (MA3-916, Affinity BioReagents Inc) in 0.5 ml of 62.5 mmol/L Tris-HCl buffer (pH 7.5), 0.9% NaCl, 0.5mmol/L NaF, 1% Triton-X, and protease inhibitors for 2 hours at 4°C. The samples were then incubated with protein G Sepharose beads (Sigma) overnight at 4°C, after which the beads were washed five times with CaMKII phosphorylation buffer (50 mol/L Tris-HCl, 10 mmol/L MgCl₂, 2 mmol/L DDT, 0.1 mmol/L Na₂EDTA, pH 7.5). CaMKII 250 U (New England Biolabs) was activated with 100 µmol/L ATP, 1.2 µmol/L calmodulin and 0.2 mmol/L CaCl₂. CaMKII phosphorylation of immunoprecipitated RyR2 was initiated by addition of autophosphorylated CaMKII and \(\gamma\)P\(^{32}\)-ATP to a final specific activity of 300 µCi/µmol. The reaction was terminated with 5 µl of stop solution (10% SDS, 300 mM EGTA and 0.25 mM DTT) after incubation for different times. Samples were heated to 95°C, size-fractionated on 5% SDS-PAGE, exposed to film and RyR2 radioactivity was quantified using Unscan it software. The amount of RyR2 protein was determined by immunoblotting with RyR antibody.

**FKBP Co-Immunoprecipitation with RyR2**

Myocytes from both WT and PLB-KO mice were treated in three conditions: 1) no treatment, as control group; 2) incubation of permeabilized myocytes (by saponin 100µg/ml) with PKA catalytic subunit (1000 units/ml, cat # 539481, Calbiochem) for 5 min to mimic functional experiments; 3) incubation of permeabilized myocytes with PKI (15 µM, cat # 116805, Calbiochem) for 5 min. Reactions were terminated by adding solubilization buffer and then snap-frozen in liquid nitrogen and stored at -80°C. Homogenates were treated in a similar way. Total FKBP were measured as described by using anti-FKBP12 (1:1000, a gift from Dr. A.R. Marks, Columbia University, New York). Proteins were transferred to PVDF membranes, and blocked with 5% milk and 0.05% Tween 20 in PBS. After incubation with secondary antibodies, immunoreactive proteins were detected with enhance chemiluminescence (ECL, from Pierce) and proteins were quantified. For FKBP studies RyR2 channels were immunoprecipitated with antibody (MA3-925, Affinity BioReagents Inc) from 500 µg of homogenate or cell lysate in 0.5 ml of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 1% NP40, 1% Na-deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF) and protease inhibitors overnight at 4°C. Samples were then incubated with protein G Sepharose beads for 2h at 4°C, after which the beads were washed 3 times with RIPA buffer. Samples were split in half and run on both 15% polyacrylamide gels (immunoblotted by anti-FKBP) and on 6% gels for measurement of RyR.

**Data Analysis**

Ca\(^{2+}\) spark were analyzed as previously described. Briefly, Ca\(^{2+}\) spark amplitudes were normalized (F/F₀) to fluorescence baseline (F₀). Ca\(^{2+}\) spark duration was the full-duration half-maximum (FDHM) and width was the full-width half-maximum (FWHM). Ca\(^{2+}\) spark frequency (CaSpF) was normalized to time and spatial size of the volume elements (or voxels) of the cell from which Ca sparks are measured. It is based on point spread function of the confocal microscope. Under our conditions (zoom = 3.0), the width and length of one voxel is 0.2 µm & 0.2 µm, and the depth (assumed) is 1 µm, so the voxel is 0.04 µm\(^3\). The fluorescence images were recorded in longitudinal line-scan mode (512 pixels per line), such that the volume of one scan line corresponds to 0.04 µm\(^3\) times the number of pixels that are within the cell length. Results are expressed as mean±SEM. Significance (P<0.05) was determined using Student’s t test.
ADDITIONAL RESULTS AND INTERPRETATIONS

Use of different [Ca\textsuperscript{2+}]i for WT and PLB-KO
The rationale for using 50 nM [Ca] in WT and 10-25 nM in PLB-KO was to have CaSpF of comparable range in PLB-KO vs. WT myocytes. Basal CaSpF was still higher in PLB-KO. This is because SERCA activity is greatly stimulated in PLB-KO myocytes (especially at low [Ca\textsubscript{i}]). We routinely find higher SR Ca content and CaSpF in intact myocytes from PLB-KO mice (e.g. ref 3) and SR Ca leak and CaSpF depend strongly on SR Ca content. Notably, the increase in SR Ca-ATPase activity at low [Ca\textsubscript{i}] is especially increased in the PLB-KO because of the effect on K\textsubscript{m} (see Fig S1 at right; modified from Fig 85 in ref 4). In particular at 30 nM in this graph SR Ca-ATPase is increased by ~8 fold higher in PLB-KO vs WT. Indeed, the SR Ca uptake rate at 25 nM in PLB-KO is nearly 3 times that for WT at 50 nM. This, we think readily explains the higher basal CaSpF in PLB-KO (in Fig 1B vs 2B and 3A vs 3B, and our prior studies). Note that when 10 nM was used in PLB-KO in Fig 5 (vs. 25 nM in Fig 1-3), the CaSpF was lower and there was less difference between PLB-KO and WT with respect to basal CaSpF.

Why isn’t SR Ca content reduced by enhanced CaSpF in PLB-KO?
We were somewhat surprised that a significant decrease in SR Ca content was not seen in PLB-KO myocytes after CaMKII activation, due to the large enhancement of CaSpF (i.e. SR Ca leak). The likely explanation is as follows. In the PLB-KO, SR Ca-pump activity is especially high, and under our conditions (low [Ca\textsubscript{i}]) SR Ca\textsuperscript{2+} leak rates are rather low. As such the SR Ca-ATPase may better approach its thermodynamic limit (i.e. the maximal [Ca\textsubscript{SR}]/[Ca\textsubscript{i}] gradient allowed by energy available to the SR Ca-ATPase (ΔG\textsubscript{ATP}). That is, the pump rate may be so much higher than the leak rate that the content is little altered by the leak rate, even if it is increased somewhat (see ISO/PLB-KO curve in Fig S2; modified from Ref 4: Fig 91, pg 176).

Are PLB-KO mice more “on the edge”? 
Certain CaMKII-induced differences were more prominent or obvious in PLB-KO than they were in WT myocytes. This includes Ca spark properties (Table 1), CaSpF during CaM/CaMKII exposure (Fig 1B vs. 2B), enhancement of Ca sparks by high free [CaM] (which was CaMKII-dependent, Fig 3B) and appearance of macrosparks and mini-waves (Fig 6). All of these things may be related to the higher SR Ca content and basal CaSpF in PLB-KO vs. WT myocytes. This puts the PLB-KO on the steep part of the SR Ca leak vs. Load relationship (see Fig S3, modified from actual data\textsuperscript{5}). In this sense the PLB-KO myocytes may be more perched “on the edge” of the steep part of the curve, allowing the changes to be more apparent upon CaMKII activation.

References

