Sarcoplasmic Reticulum Ca$^{2+}$ Refilling Controls Recovery From Ca$^{2+}$-Induced Ca$^{2+}$ Release Refractoriness in Heart Muscle

Peter Szentesi, Christophe Pignier, Marcel Egger, Evangelia G. Kranias, Ernst Niggli

Abstract—In cardiac muscle Ca$^{2+}$-induced Ca$^{2+}$ release (CICR) from the sarcoplasmic reticulum (SR) is initiated by Ca$^{2+}$ influx via L-type Ca$^{2+}$ channels. At present, the mechanisms underlying termination of SR Ca$^{2+}$ release, which are required to ensure stable excitation-contraction coupling cycles, are not precisely known. However, the same mechanism leading to refractoriness of SR Ca$^{2+}$ release could also be responsible for the termination of CICR. To examine the refractoriness of SR Ca$^{2+}$ release, we analyzed Na$^+$-Ca$^{2+}$ exchange currents reflecting cytosolic Ca$^{2+}$ signals induced by UV-laser flash-photolysis of caged Ca$^{2+}$. Pairs of UV flashes were applied at various intervals to examine the time course of recovery from CICR refractoriness. In cardiomyocytes isolated from guinea-pigs and mice, β-adrenergic stimulation with isoproterenol-accelerated recovery from refractoriness by ≈2-fold. Application of cyclopiazonic acid at moderate concentrations (<10 μmol/L) slowed down recovery from refractoriness in a dose-dependent manner. Compared with cells from wild-type littersmates, those from phospholamban knockout (PLB-KO) mice exhibited almost 5-fold accelerated recovery from refractoriness. Our results suggest that SR Ca$^{2+}$ refilling mediated by the SR Ca$^{2+}$-pump corresponds to the rate-limiting step for recovery from CICR refractoriness. Thus, the Ca$^{2+}$ sensitivity of CICR appears to be regulated by SR Ca$^{2+}$ content, possibly resulting from a change in the steady-state Ca$^{2+}$ sensitivity and in the gating kinetics of the SR Ca$^{2+}$ release channels (ryanodine receptors). During Ca$^{2+}$ release, the concomitant reduction in Ca$^{2+}$ sensitivity of the ryanodine receptors might also underlie Ca$^{2+}$ spark termination by deactivation. (Circ Res. 2004;95:807-813.)

Key Words: calcium-induced calcium release ▪ excitation-contraction coupling ▪ cardiac myocyte ▪ heart ▪ sarcoplasmic reticulum ▪ calcium content ▪ photolysis of caged calcium

The contraction of ventricular myocytes is the final step of excitation-contraction (EC) coupling in heart muscle. During this process, Ca$^{2+}$ entering via L-type Ca$^{2+}$ channels induces a massive liberation of sarcoplasmic Ca$^{2+}$ (Ca$^{2+}$-induced Ca$^{2+}$ release [CICR]) through Ca$^{2+}$ release channels (ryanodine receptors [RyRs]). CICR occurs by a concerted activation of numerous Ca$^{2+}$-release events (Ca$^{2+}$ sparks). Relaxation requires both the termination of Ca$^{2+}$ sparks and the removal of Ca$^{2+}$ from the cytosol. Mechanisms terminating Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) are presently not well characterized, but several possibilities have been discussed. These include Ca$^{2+}$-induced inactivation of RyRs, adaptation of RyRs, stochastic attrition, and functional depletion of the SR Ca$^{2+}$ stores. A substantial body of indirect experimental evidence indicates that one or more mechanisms leading to refractoriness of the CICR must exist. However, studies examining the time course of recovery of cardiac force after a twitch contraction have generally failed to detect a refractoriness of CICR. But a genuine and slowly recovering refractoriness of CICR may be difficult to detect with a technique that relies on the Ca$^{2+}$ current, because this trigger itself exhibits a recovery from inactivation. Using a different approach, we have recently observed that activation of SR Ca$^{2+}$ release by UV-laser flash photolysis of caged Ca$^{2+}$ indeed unmask a genuine refractoriness of CICR. With photochemically generated Ca$^{2+}$-concentration jumps, CICR can be triggered independently of L-type Ca$^{2+}$ currents. We then suggested that Ca$^{2+}$ store depletion may underlie, at least in part, the observed refractoriness, and that SR Ca$^{2+}$ content may determine the Ca$^{2+}$ sensitivity of the RyRs on the cytosolic side of the channels.

The goal of the present study was to examine whether SR Ca$^{2+}$ content may indeed determine RyR Ca$^{2+}$ sensitivity and whether SR Ca$^{2+}$ refilling is the rate-limiting step determining the recovery from refractoriness. For this purpose, we applied pharmacological interventions to correlate SR Ca$^{2+}$

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807
pump (SERCA) activity with the time course of recovery from refractoriness. To this end, we elicited Ca$^{2+}$ release from the SR by using UV-laser flash photolysis of caged Ca$^{2+}$ as the trigger. The resulting Na$^+$-Ca$^{2+}$ exchange currents ($I_{\text{NCX}}$) were analyzed to evaluate SR Ca$^{2+}$ release events and to characterize the refractoriness of CICR in terms of its temporal features. Analogous experiments were also performed in myocytes from phospholamban (PLB)-deficient mice. PLB is a protein, which regulates SR Ca$^{2+}$ cycling in cardiomyocytes by reversibly inhibiting the SERCA. We found that SR Ca$^{2+}$ refilling is strongly influencing recovery from CICR refractoriness. Thus, the Ca$^{2+}$ sensitivity of the RyRs appears to be regulated by SR Ca$^{2+}$ content. Preliminary reports describing this work have been presented in abstract form.12,13

Materials and Methods

Cell Isolation

All experiments were performed according to the guidelines of the Swiss Animal Protection Law and with the permission of The State Veterinary Office, Bern, Switzerland. Cardiac ventricular myocytes were isolated from adult guinea pigs and mice (Schneider, Rubigen, Switzerland) using established enzymatic procedures.14 The superfusion solution contained (in mmol/L): 140 NaCl, 5 KCl, 0.5 BaCl$2$, 1.8 CaCl$2$, 1 CsCl, 10 HEPES, 10 glucose, pH 7.4 (NaOH). The pipette filling solution contained (in mmol/L): 120 Cs-aspartate, 20 tetraethylammonium chloride, 2 Na$_4$-DM-nitrophen (1-(2-nitro-4,5-dimethoxyphenyl)-1,2-diaminoethane-N,N,N',N'-tetraacetic acid, 4Na$^+$), 2 reduced glutathione, 0.05 Fluo-3, 0.5 CaCl$2$, 5 K$_2$-ATP, 20 HEPES, pH 7.2 (CsOH). In experiments with mouse cells, the Na$_4$-DM-nitrophen concentration was 4 mmol/L. All experiments were performed at room temperature (20°C to 22°C). Where required, the superfusion solution contained isoproterenol (1 mmol/L; Sigma) or cyclopiazonic acid (1, 5 or 10 μmol/L; Sigma).

Electrophysiological Recordings

Cells were voltage-clamped in the whole-cell configuration of the patch-clamp technique and held at −80 mV. The SR was loaded with Ca$^{2+}$ by six depolarizing steps to 10 mV lasting 200 ms, at 1 Hz, to elicit L-type Ca$^{2+}$ currents. $I_{\text{NCX}}$ were recorded at −40 mV to estimate changes in intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]). Within the [Ca$^{2+}$], jumps reached under our conditions, $I_{\text{NCX}}$ is known to depend linearly on [Ca$^{2+}$].1,15 The SR Ca$^{2+}$ content was estimated by integrating the $I_{\text{NCX}}$ induced by rapid application of 20 mmol/L caffeine. All values are expressed as means±SEM. Student unpaired t tests were used to test for statistical significance (P<0.01, as indicated).

Flash Photolysis

Pairs of UV-light flashes were used to photolyse intracellular DM-nitrophen in an epi-illumination arrangement and were generated with a frequency-tripled neodymium: yttrium-aluminum garnet laser (Nd:YAG laser) (wavelength 355 nm, maximal energy 20.7 mJ, repetition rate up to 10 Hz; Shurelite II; Continuum). Photoconsumption of DM-nitrophen in our setup was determined as ~4% at maximum tolerable laser power.

Results

UV-laser flash photolysis of caged Ca$^{2+}$ was used to activate CICR with a paired-pulse protocol. This approach allowed us to examine the refractoriness of SR Ca$^{2+}$ release and to determine the time course of recovery from refractoriness. As we have shown before, this technique has the advantage that two trigger signals of identical amplitude can be applied to unmask and analyze the CICR refractoriness.9 For the experiments shown below, the photolytic triggers have the additional advantage of remaining constant during β-adrenergic stimulation of the myocytes, unlike the L-type Ca$^{2+}$ currents, which would increase in amplitude.

β-Adrenergic Stimulation Accelerates Recovery From Refractoriness

In the following experiments, we used guinea pig ventricular myocytes to record $I_{\text{NCX}}$ generated by the electrogenic Na$^+$-Ca$^{2+}$ exchanger, as estimates of changes in the intracellular Ca$^{2+}$ concentration, [Ca$^{2+}$]. $I_{\text{NCX}}$ has been shown to be linearly dependent on [Ca$^{2+}$], over the range of concentrations covered by UV-flash photolysis of caged Ca$^{2+}$.9,15 Laser-flash photolysis of DM-nitrophen caused a rapid and homogeneous Ca$^{2+}$ concentration jump (τ<30 μs) that resulted in an almost immediate activation of inward $I_{\text{NCX}}$.16 The fast photochemical Ca$^{2+}$ release from DM-nitrophen was reflected in a rapidly rising $I_{\text{NCX}}$ component, followed by a slower increase in $I_{\text{NCX}}$ ($I_{\text{NCX,slow}}$), continuing for ~50 ms before $I_{\text{NCX}}$ started to decline, in parallel with the decrease in [Ca$^{2+}$]. The slow component of $I_{\text{NCX}}$ activation arises from CICR triggered by the photolytic Ca$^{2+}$ release, and the amplitude of $I_{\text{NCX,slow}}$ can be used to estimate the magnitude of CICR (see also inset in Figure 1Ba).9 Figure 1A shows the loading protocol, which was applied to ensure comparable SR Ca$^{2+}$ load conditions. In this series of experiments, we applied isoproterenol as an agonist for the β-adrenergic receptors to accelerate the SERCA. Subsequent to receptor activation, Protein kinase A–dependent phosphorylation of PLB is known to relieve the suppression of the SERCA, thereby, accelerating the pump and SR Ca$^{2+}$ refilling. Thus, we used the SERCA stimulation to test the hypothesis that recovery from CICR refractoriness is governed by SR Ca$^{2+}$ refilling.

Figure 1C compares the results from experiments with pairs of UV-laser flashes in control conditions (left panels) and in the presence of 1 μmol/L isoproterenol (right panels). The concentration of isoproterenol used was ~100-fold the EC$50$ and was chosen to rapidly activate a large fraction of the β-adrenergic receptors.17 When the interval between successive flashes was longer than ~500 ms, $I_{\text{NCX}}$ exhibited approximately the same peak amplitude, particularly in the presence of β-adrenergic stimulation (Figure 1C). However, when the interval between photolytic trigger signals was gradually reduced, the second $I_{\text{NCX}}$ in a pair became progressively smaller in both cases (Figure 1C and 1D). Previous experiments in the presence of ryanodine and thapsigargin have confirmed that the photolytic component and, therefore $I_{\text{NCX}}$ per se, was not smaller after the second flash.9 (An example is also shown in Figure 1Bb.) The reduction of the slow component of $I_{\text{NCX}}$ at short intervals thus indicates that the CICR was curtailed and refractory after the second pulse of a pair (see inset, Figure 1B). In control conditions, SR Ca$^{2+}$ release exhibited refractoriness recovering with a τ of 717.9±60.9 ms (Figure 1D). Interestingly, the $I_{\text{NCX}}$ decayed faster with a τ of 162.4±13.8 ms. Even though this time course reflects the decay of [Ca$^{2+}$] near the sarcolemma, it allows an, albeit very indirect, estimate of SR refilling. The recovery of CICR was significantly accelerated by isoproterenol treatment (τ=342.0±15.8 ms). Although this result is
entirely consistent with our hypothesis, unfortunately, β-adrenoceptor stimulation is thought to alter many cellular processes involved in cardiac Ca\(^{2+}\) signaling, including RyR phosphorylation and changes of steady-state SR Ca\(^{2+}\) content via the intended SERCA stimulation.\(^{18-21}\) Both mechanisms could, in principle, lead to modifications of the Ca\(^{2+}\) sensitivity of the RyRs and thus of CICR, which might change the time course of recovery from CICR refractoriness independently of the rate of SR refilling.

Inhibition of the SERCA Slows Recovery From CICR Refractoriness

Because the accelerated recovery from refractoriness after β-adrenoceptor stimulation could result from several modifications of the Ca\(^{2+}\)-signaling machinery, we intended to apply a more specific pharmacological approach to test the hypothesis that SERCA activity governs CICR recovery. To slow down the SERCA several inhibitors are available. Cyclopiazonic acid (CPA), an inhibitor of the SERCA, could be a pharmacological tool to tune its function in a graded fashion.\(^{22}\) In SR vesicles derived from cardiac muscle, CPA inhibits Ca\(^{2+}\) uptake at nanomolar concentrations. In contrast to thapsigargin, the inhibitory effect of CPA is reversible. We applied CPA at low concentrations to slow down, but not completely inhibit, the SERCA. This would be expected to subsequently slow down the recovery from CICR refractoriness, at least when SR Ca\(^{2+}\) refilling via the SERCA was the rate-limiting step. Before carrying out experiments on the recovery from CICR refractoriness, we assessed the steady-state SR Ca\(^{2+}\) content during application of CPA. The voltage-clamp protocol outlined in Figure 1A was applied, and the SR was again loaded Ca\(^{2+}\) by applying a train of six I_{Ca,L}. But instead of using UV flashes after the loading protocol, the cells were briefly exposed to 20 mmol/L caffeine to empty the SR. The amount of Ca\(^{2+}\) released by the application of caffeine was estimated by integrating the caffeine-induced I_{NCX} recorded at −40 mV. Figure 2A shows the relative SR Ca\(^{2+}\) content after 1 minute application of 1 μmol/L, 5 μmol/L, and 10 μmol/L CPA. The lowest concentration of CPA used is smaller than the IC\(_{50}\) in intact cells and was selected to inhibit just a fraction of the SERCAs. It has been shown previously that this concentration decreases ≈25% decrease in the twitch tension of guinea pig atria.\(^{23}\) In the presence of CPA the SR Ca\(^{2+}\) content decreased in a concentration-dependent manner. The data revealed that these CPA concentrations were sufficient to gradually slow down the SERCA function and subsequently lower the SR Ca\(^{2+}\) content. To verify that we could still release Ca\(^{2+}\) from the SR and thus activate CICR with flash photolytic Ca\(^{2+}\) concentration jumps, we determined the ratio of CICR (I_{NCX,slow}) before drug application and in the presence of CPA. Figure 2B shows that SR Ca\(^{2+}\) release could still be elicited photolytically despite a 1 minute exposure to a low concentration of CPA.

Recovery From CICR Refractoriness in the Presence of CPA

To assess the effect of slowing down (but not completely inhibiting) Ca\(^{2+}\) uptake via the SERCA, we performed refrac-
A CPA concentration of 10 μmol/L almost completely inhibited the SERCA (Figure 3, inset), similar to our previous observations with thapsigargin (not shown). This high CPA concentration slowed it down, as expected because of the abbreviated Ca\(^{2+}\)-induced inactivation present in these animals. The application of isoproterenol and CPA in WT mice resulted in changes of the time course of recovery similar to those observed in guinea pigs and WT mice (Figure 4B: PLB-KO cells). As expected, β-adrenergic stimulation could not further accelerate the recovery in PLB-KO mice, whereas CPA slowed it down, as in guinea pigs and WT mice (Figure 4B: PLB-KO cells). This high CPA concentration thus prevented sufficient refilling of the SR within the time window of our experiments.

### Discussion

EC-coupling in cardiac muscle is inherently unstable because of its positive feedback arising from CICR. However, to ensure stable EC-coupling behavior, one or more mechanisms terminating Ca\(^{2+}\) release from the SR are required. To date, it is not clear to what extent Ca\(^{2+}\)-induced inactivation or adaptation of RyRs, stochastic attrition, or functional depletion of the SR Ca\(^{2+}\) stores contribute and interact to terminate SR Ca\(^{2+}\) release. However, Ca\(^{2+}\)-induced inactivation of RyRs has been difficult to demonstrate in intact cells, presumably because it may only occur at the very high Ca\(^{2+}\) concentrations prevailing in the microenvironment of the

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**Figure 2.** Effects of CPA on SR Ca\(^{2+}\) content and SR Ca\(^{2+}\) release. A, SR Ca\(^{2+}\) content determined by integrating (over time, dt) \(I_{\text{CICR}}\) induced by rapid caffeine application (20 mmol/L) in control and in the presence of 1, 5, and 10 μmol/L CPA (64.1±5.7%, n=14; 47.1±7.5%, n=10; and 38.7±4.6%, n=18, respectively). B, SR Ca\(^{2+}\) release obtained from the CICR component of \(I_{\text{CICR(slow)}}\) in control and in the presence of 1, 5, and 10 μmol/L CPA triggered by a single UV-Flash (83.5±4.8%, n=21; 76.4±5.2%, n=11; and 63.5±5.4%, n=15, respectively).

**Figure 3.** Slowing SR Ca\(^{2+}\) refilling with CPA. Averaged data showing the recovery from CICR refractoriness under control conditions (○) and in the presence of 1 μmol/L (■) and 5 μmol/L (□) CPA. Solid lines are the least-square fit of a mono-exponential function to the averages (control, \(τ=720.0±31.8\) ms; 1 μmol/L CPA, \(τ=792.2±23.6\) ms; 5 μmol/L CPA, \(τ=943.9±34\) ms; mean±SEM, n=5 to 27). The inset shows the effects of 10 μmol/L CPA (■) on recovery from CICR refractoriness.

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**Effect of Phospholamban on Recovery from CICR Refractoriness in Genetically Modified Mice**

Both the acceleration of CICR recovery after β-adrenergic stimulation and the retardation of recovery during SERCA inhibition are consistent with the notion that SR refilling plays an important role for the time course of recovery from refractoriness. To validate this conclusion we wanted to make use of an approach not entirely relying on pharmacological tools. To this end, we performed refractoriness experiments on myocytes isolated from PLB knockout (PLB-KO) mice and their respective wild-type (WT) littermates. The experiments were analogous to those described in Figures 1 and 3. The left column of Figure 4 (A and B) shows examples of exchange currents elicited by pairs of UV-laser flashes with a shorter (200 ms) and a longer (900 ms) interval recorded in myocytes from WT (Figure 4A) and PLB-KO (Figure 4B) mice. Compared with \(I_{\text{CICR}}\) from WT, the traces recorded from PLB-KO mice showed a more rapid decay, as expected because of the abbreviated Ca\(^{2+}\) transient present in these animals. The application of isoproterenol and CPA in WT mice resulted in changes of the time course of recovery similar to those observed in guinea pigs (Figure 4A: control, 239.5±8.2 ms; isoproterenol, 175.5±7.1 ms; CPA, 531.3±9.4 ms). Compared with WT myocytes, the recovery was accelerated almost 5-fold in PLB-KO cells (Figure 4B). As expected, β-adrenergic stimulation could not further accelerate the recovery in PLB-KO mice, whereas CPA slowed it down, as in guinea pigs and WT mice (Figure 4B: PLB-KO cells). This high CPA concentration thus prevented sufficient refilling of the SR within the time window of our experiments.

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**Figure 4.** Averaged data showing the recovery from CICR refractoriness under control conditions (○) and in the presence of 1 μmol/L (■) and 5 μmol/L (□) CPA. Solid lines are the least-square fit of a mono-exponential function to the averages (control, \(τ=720.0±31.8\) ms; 1 μmol/L CPA, \(τ=792.2±23.6\) ms; 5 μmol/L CPA, \(τ=943.9±34\) ms; mean±SEM, n=5 to 27). The inset shows the effects of 10 μmol/L CPA (□) on recovery from CICR refractoriness.
dyadic cleft.\textsuperscript{28,29} Also the recovery from CICR refractoriness has been difficult to examine because of the temporal overlap of this process with the recovery of $I_{Ca,L}$ from inactivation.\textsuperscript{8,29}

However, recently we were able to unmask a genuine and slowly recovering refractoriness of CICR when activating SR Ca$^{2+}$ release by UV-laser flash photolysis of caged Ca$^{2+}$.\textsuperscript{9} This technique produces a coherent and homogeneous trigger for CICR and is an ideal approach to examine refractoriness of CICR because it bypasses $I_{Ca,L}$, thus avoiding inactivation of the trigger signal and effects of isoproterenol on $I_{Ca,L}$.

Based on the observed discrepancies between slow global and fast local recovery from CICR refractoriness, we proposed that the SR Ca$^{2+}$ content may play a regulatory role for the Ca$^{2+}$ sensitivity of the RyRs. In other words, if Ca$^{2+}$-induced inactivation (or adaptation) of RyRs would be the dominant mechanism for CICR refractoriness, then it should be seen not only in global but also in localized CICR events. Interestingly, slowly recovering refractoriness was not observed on the level of local Ca$^{2+}$-release signals. Because both inactivation and adaptation are even functioning on the level of a single RyR channel, they do not require activation of the entire SR network. This conclusion is also supported by our observation of flash-photolytically induced alternans of Ca$^{2+}$ release.\textsuperscript{3} This type of alternans implies that the rise of cytosolic Ca$^{2+}$ concentration alone is insufficient to inactivate the RyRs. Instead, release of Ca$^{2+}$ from the SR is required to terminate CICR and cause refractoriness. Therefore, we proposed that recovery from CICR refractoriness is determined by the time course of SR Ca$^{2+}$ refilling subsequent to global Ca$^{2+}$ release.\textsuperscript{9} In principle, two scenarios can be imagined how SR Ca$^{2+}$ refilling could relieve refractoriness: (1) the SR Ca$^{2+}$ concentration could directly affect the RyR Ca$^{2+}$ sensitivity and thus the recovery from refractoriness by binding to a specific site within the SR; and (2) additional rate-limiting steps could be required after SR Ca$^{2+}$ refilling, such as conformational changes of one or more proteins.

In the present study, we successfully distinguished between these two possibilities by examining how luminal SR Ca$^{2+}$ content can control recovery from CICR refractoriness. When Ca$^{2+}$ inside the SR exerts its effect on the RyR Ca$^{2+}$ sensitivity without subsequent rate-limiting steps, modifications of the SERCA activity should result in immediate changes in the rate of recovery from CICR refractoriness, which was observed. Nevertheless, some additional, but not rate-limiting, steps may be required after SR refilling (see below). As expected for an immediate effect, acceleration of the SERCA by isoproterenol hastened the recovery from CICR refractoriness. Unfortunately, \( \beta \)-adrenergic stimulation is not specific for the SERCA, and this observation could arise from protein kinase A–mediated phosphorylation of various proteins other than PLB.\textsuperscript{18–21} To clarify this question, we initially tried to target the SERCA function more specifically with thapsigargin. Unfortunately, SERCA inhibition by thapsigargin led to almost no recovery from refractoriness, probably as a result of an excessive inhibition of the SERCA or a reduction of the SR Ca$^{2+}$ content below a level critical for CICR.\textsuperscript{30} However, the use of low and intermediate concentrations of CPA permitted better control over SERCA inhibition. At high concentrations of CPA, the recovery from CICR refractoriness was again minimal and similar to that observed in the presence of thapsigargin. Even the first flash did not trigger a substantial SR Ca$^{2+}$ release; thus CICR already exhibited a state similar to refractoriness, probably attributable to reduced SR Ca$^{2+}$ content. This was confirmed by the application of caffeine puffs, which revealed that the SR contained significantly less Ca$^{2+}$ in presence of high concentrations of CPA. However, at low concentrations of CPA, recovery from CICR refractoriness was slowed down in a dose-dependent manner. In the presence of CPA the
recovery from CICR refractoriness remained incomplete, as expected. This may have several reasons: (1) significant photoconsumption of the caged compound which is not replenished from the patch-pipette between two flashes leads to a reduced second $I_{\text{CXC}}$ (2) in the presence of CPA, the SR may not refill to the same extent after a flash as during the vigorous SR loading protocol with several Ca$^{2+}$ currents; or (3) there may be also a slower phase of recovery of some RyRs, which could take several seconds.\textsuperscript{31}

In experiments performed in myocytes obtained from PLB-KO mice, we found an almost 5-fold acceleration of recovery from refractoriness, when compared with WT myocytes. PLB is known to suppress the SERCA, and the ablation of PLB has been described to accelerate SR refilling.\textsuperscript{31,32} The lack of any further acceleration of recovery from refractoriness by exposure to isoproterenol in PLB-KO mice suggests that SERCA activation and subsequent changes of SR Ca$^{2+}$ refilling are the dominant effects of $\beta$-adrenergic stimulation under our experimental conditions. However, this observation does not allow for the inference that phosphorylation of proteins other than PLB (eg, the RyRs) has no effect on CICR features other than the amplitude of the transient (eg, on the gating cooperativity or open probability of the RyRs). Based on our results, we conclude that Ca$^{2+}$ refilling of the SR via the SERCA is a rate-limiting step governing recovery from CICR refractoriness. However, $I_{\text{CXC}}$ signals reflecting subsarcolemmal [Ca$^{2+}$]$\text{$_{i}$}$ and, indirectly, SR refilling, decay more rapidly than recovery of CICR occurs. This may reflect subsarcolemmal Ca$^{2+}$ depletion resulting from the Na$^{+}$–Ca$^{2+}$ exchange activity,\textsuperscript{33} but could also be explained by one or more additional steps required for the recovery of CICR subsequent to SR refilling. Taken together, the most likely explanation for our observations is that CICR refractoriness results from SR Ca$^{2+}$ depletion during Ca$^{2+}$ release and that store emptying leads to deactivation and termination of the Ca$^{2+}$ release by reducing the Ca$^{2+}$ sensitivity of the RyRs on the cytosolic side of the channels.\textsuperscript{34} This notion is also supported by recent findings of prolonged Ca$^{2+}$ spark durations after increasing the buffer capacity and Ca$^{2+}$ content of the SR, either by adding chemical buffers or by overexpression of calsequestrin.\textsuperscript{27,35}

An important unsolved question is how the EC-coupling machinery can sense the releasable SR Ca$^{2+}$ content or the free Ca$^{2+}$ concentration in the SR. In any case, SR Ca$^{2+}$ content–dependent regulation and termination of CICR requires the presence of Ca$^{2+}$ sensors inside the SR or on the luminal side of the RyRs. In bilayer experiments, the presence of Ca$^{2+}$-binding sites on the luminal side of the RyR has been reported\textsuperscript{16} (but this notion has not remained unchanged\textsuperscript{37}). Alternatively, this signaling pathway may involve Ca$^{2+}$ binding to proteins inside the SR, like calsequestrin or other Ca$^{2+}$-binding proteins. This in turn may induce conformational changes, which are relayed to the RyRs via allosteric interactions and/or via other proteins, such as triadin and junctin.\textsuperscript{38,39} This finally could lead to a change in the sensitivity of the RyRs for Ca$^{2+}$.\textsuperscript{25} In fact, very recently it was shown that by adding calsequestrin, triadin, and junctin to purified RyRs incorporated in lipid bilayers, the dependence of the channels on luminal Ca$^{2+}$ can be restored.\textsuperscript{40} In the future, additional studies in intact cells will be required to identify and characterize the signaling pathway that connects local and global SR Ca$^{2+}$ content with the Ca$^{2+}$ sensitivity of the RyRs for incoming trigger signals. A detailed understanding of the mechanisms regulating RyR Ca$^{2+}$ sensitivity is not only a prerequisite for comprehending EC-coupling, but also for appreciating the importance of pathological alterations in EC-coupling during diseases.\textsuperscript{41} In addition, disruption of the normal function of SR Ca$^{2+}$ buffering has recently been reported to be causally related to potentially lethal arrhythmias.\textsuperscript{42}

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