Potentiation of Ca^{2+} Release by cADP-Ribose in the Heart Is Mediated by Enhanced SR Ca^{2+} Uptake Into the Sarcoplasmic Reticulum

Valeriy Lukyanenko, Inna Györke, Theodore F. Wiesner, Sandor Györke

***Abstract*—**cADP-Ribose (cADPR) is a novel endogenous messenger that is believed to mobilize Ca^{2+} from ryanodine-sensitive Ca^{2+} stores. Despite intense research, the precise mechanism of action of cADPR remains uncertain, and experimental findings are contradictory. To elucidate the mechanism of cADPR action, we performed confocal Ca^{2+} imaging in saponin-permeabilized rat ventricular myocytes. Exposure of the cells to cADPR resulted in a slow (>2 minutes) and steady increase in the frequency of Ca^{2+} sparks. These effects on local release events were accompanied by a significant increase in sarcoplasmic reticulum (SR) Ca^{2+} content. In comparison, sensitization of ryanodine receptors (RyRs) by caffeine, a true RyR agonist, caused a rapid (<1 second) and transient potentiation of Ca^{2+} sparks followed by a decrease in SR Ca^{2+} content. When the increase in the SR load was prevented by partial inhibition of the SR Ca^{2+} with thapsigargin, cADPR failed to produce any increase in sparking activity. cADPR had no significant impact on activity of single cardiac RyRs incorporated into lipid bilayers. However, it caused a significant increase in the rate of Ca^{2+} uptake by cardiac SR microsomes. Our results suggest that the primary target of cADPR is the SR Ca^{2+} uptake mechanism. Potentiation of Ca^{2+} release by cADPR is mediated by increased accumulation of Ca^{2+} in the SR and subsequent luminal Ca^{2+}-dependent activation of RyRs. **(*Circ Res*. 2001;89:614-622.)**

Key Words: ventricular myocytes ■ ryanodine receptors ■ sarcoplasmic reticulum Ca^{2+} ■ Ca^{2+} sparks ■ cADP-ribose

In mammalian cardiac myocytes, contractile activation occurs in response to Ca^{2+} influx from the extracellular space. This triggers Ca^{2+}-induced Ca^{2+} release via Ca^{2+} release channels known as ryanodine receptors (RyRs), which reside in the membrane of the sarcoplasmic reticulum (SR).[1,2] The function of cardiac RyRs has been studied extensively by monitoring global and local intracellular Ca^{2+} release signals with fluorescent Ca^{2+} indicators, by measuring Ca^{2+} fluxes from isolated SR preparations, and by recording single-channel currents by using the planar lipid bilayer technique.[3] These studies have shown that the activity of the RyR is controlled by a number of endogenous ligands, including Ca^{2+} in both the cytosol and the SR lumen, ATP, Mg^{2+}, and calmodulin. Consequently, the RyR is thought to carry the corresponding binding sites.

In recent years, cADP-ribose (cADPR) has emerged as a potential endogenous regulator of RyR activity.[4] Despite intense research, the precise mechanism of action of cADPR remains uncertain, and experimental findings are contradictory. Early in vitro studies reported that cADPR can directly activate RyRs reconstituted into lipid bilayers.[5] Subsequent studies, however, indicated no effects or detected changes that would be expected to be abolished in the presence of physiological concentrations of ATP.[6,7] On the other hand, most experiments performed in cardiac cells yielded results that are consistent with a role for cADPR in the modulation of SR Ca^{2+} release. In guinea pig ventricular cells, the cADPR antagonists 8-amino-cADPR and 8-Br-cADPR have inhibited both depolarization-induced Ca^{2+} transients and spontaneous Ca^{2+} waves.[8,9] It has been further demonstrated that cADPR applied to the cytosol increases cell-average Ca^{2+} transients and contractions[10–13] and enhances the frequency of local Ca^{2+} release events (ie, sparks) in both intact and permeabilized cardiac myocytes.[11,14] The effects of cADPR on Ca^{2+} release are highly temperature dependent and relatively slow kinetically.[10,11] These observations have been interpreted as cADPR sensitizing RyRs to cytosolic Ca^{2+}, thereby enhancing Ca^{2+}-induced Ca^{2+} release. The marked temperature dependence and complex kinetics of these effects suggest that the action of cADPR on Ca^{2+} release might involve intermediate signaling steps, such as interactions of cADPR with other proteins of the RyR complex or with calmodulin.[11] However, precisely how cADPR influences the function of Ca^{2+} release channels has not been elucidated. To explore the mechanism of action of cADPR on SR Ca^{2+} release, we investigated the effects of cADPR on Ca^{2+} release

Original received May 7, 2001; revision received August 8, 2001; accepted August 21, 2001.

From the Department of Physiology (V.L., I.G., S.G.), Texas Tech University Health Sciences Center, and the Department of Chemical Engineering (T.F.W.), Texas Tech University, Lubbock.

Correspondence to Dr Sandor Györke, Texas Tech University HSC, 3601 4th St, Lubbock, TX 9430-6551. E-mail physg@ttuhsc.edu

© 2001 American Heart Association, Inc.

***Circulation Research* is available at http://www.circresaha.org**

and on accumulation by the SR in permeabilized rat ventricular myocytes. Our experiments showed that the primary target of cADPR is the SR Ca^{2+} uptake mechanism. Potentiation of Ca^{2+} release by cADPR is mediated indirectly by increased accumulation of Ca^{2+} in the SR and subsequent luminal Ca^{2+}-dependent activation of RyRs.

Materials and Methods

Confocal Microscopy

Single ventricular myocytes were obtained from adult male Sprague-Dawley rat hearts (n=33) by enzymatic dissociation.[15] The standard Tyrode's solution contained (in mmol/L) NaCl 140, KCl 5.4, $MgCl_2$ 0.5, $CaCl_2$ 1, HEPES 10, NaH_2PO_4 0.25, and glucose 5.6, pH 7.3. The cells were permeabilized by exposure to saponin (0.01% for 45 to 60 seconds).[14] The permeabilization solution contained (in mmol/L) K aspartate 100, KCl 20, MgATP 3, $MgCl_2$ 0.81 ($[Mg^{2+}]_{free}=\approx 1$ mmol/L), EGTA 0.5, phosphocreatine 10, HEPES 20, and 5 U/mL creatine phosphokinase, pH 7.2. The control experimental solution contained (in mmol/L) K aspartate 100, KCl 20, MgATP 3, EGTA 0.5, $CaCl_2$ 0.114 ($[Ca^{2+}]_{free}=\approx 100$ nmol/L), $MgCl_2$ 0.81 ($[Mg^{2+}]_{free}=\approx 1$ mmol/L), phosphocreatine 10, HEPES 20, Fluo-3 K-salt 0.03 (TefLabs), and 5 U/mL creatine phosphokinase, pH 7.2. The free $[Ca^{2+}]$ and $[Mg^{2+}]$ at given total Ca^{2+}, Mg^{2+}, ATP, and EGTA concentrations were calculated by using (WinMAXC 1.80). All chemicals were from Sigma unless otherwise specified. All experiments were performed at room temperature (21°C to 23°C). Changes in $[Ca^{2+}]$ were recorded with a Bio-Rad Laser Scanning Confocal system (MRC-1024ES, Bio-Rad Laboratories) with an Olympus 60× 1.4 N.A. objective.[16] Fluo-3 was excited by light at 488 nm (25-mW argon laser, intensity attenuated to 0.3%), and the fluorescence was acquired at wavelengths of >515 nm in the line scan mode at rate of 6.0 ms per scan. Ca^{2+} sparks were quantified by using a detection computer algorithm, and their statistics were corrected for missed events and amplitude distortions introduced by instrumental noise.[16,17] Data were expressed as means±SE. Comparisons were performed by using the paired *t*-test, and significance was defined at $P<0.05$.

Preparation of SR Membrane Vesicles

Heavy SR microsomes were isolated by differential centrifugation from the ventricles of dog heart as described previously.[15]

Lipid Bilayer Experiments

Single RyRs were reconstituted by fusing SR microsomes into planar lipid bilayers as described previously.[18] Channel incorporation was performed in solutions containing (in mmol/L) $CsCH_3SO_3$ 350, $CaCl_2$ 0.02, HEPES 20 (pH 7.4) on the cytosolic (*cis*) side of the bilayer, and $CsCH_3SO_3$ 20, $CaCl_2$ 0.02, HEPES 20 (pH 7.4) on the lumenal (*trans*) side of the bilayer. The experimental solutions contained (in mmol/L) $CsCH_3SO_3$ 350, $CaCl_2$ 0.02 ($[Ca^{2+}]_{free}=\approx 5$ μmol/L), MgATP 3, $MgCl_2$ 0.6 ($[Mg^{2+}]_{free}=\approx 0.9$ mmol/L), HEPES 20 (pH 7.4) (*cis*), and $CsCH_3SO_3$ 20, $CaCl_2$ 0.02, HEPES 20 (pH 7.4) (*trans*). Single channels were monitored at +30 mV in symmetrical Cs^+ (350 mmol/L). Single-channel currents were recorded with an Axopatch 200A (Axon Instruments) patch-clamp amplifier. Data were filtered at 2 kHz and digitized at 5 to 10 kHz. Acquisition and analysis of data were performed by using pClamp 6.01 software (Axon Instruments).

SR Ca^{2+} Uptake Measurements

Ca^{2+} uptake measurements were performed with spectrofluorometer D-Scan (PTI). We monitored $[Ca^{2+}]$ outside the membrane vesicles using the ratiometric Ca^{2+} indicator Fura 2FF (1 mmol/L, K^+ salt). The medium in the cuvette consisted of (in mmol/L): K aspartate 100, KCl 20, $MgCl_2$ 0.81, phosphocreatine 10, MgATP 3, and 5 U/mL creatine phosphokinase, pH 7.2. To inhibit the Ca^{2+} release through RyRs, the experimental medium was supplemented with 10 μmol/L ruthenium red. Membranes (0.5 to 1.0 mg of protein per milliliter) were added to the cuvette, and active Ca^{2+} uptake was initiated by the administration of 500 nmol/L Ca^{2+}.

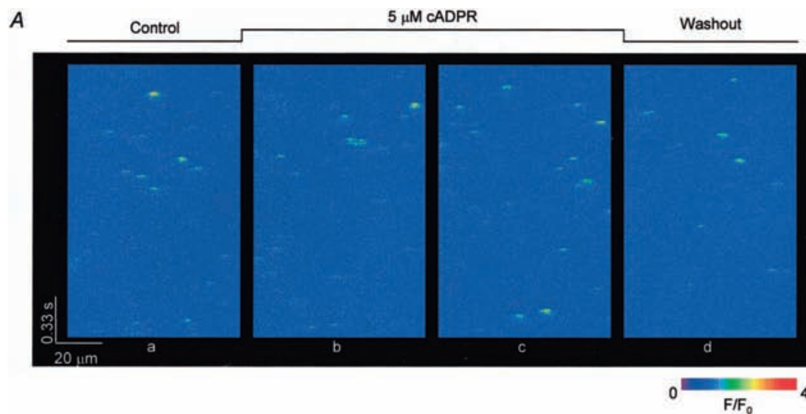
Results

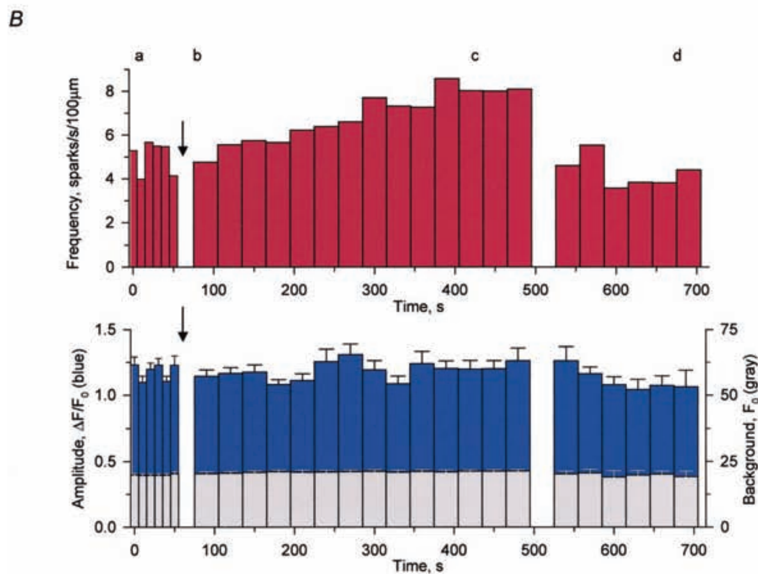
Effects of cADPR on Ca^{2+} Sparks

In accordance with our previous studies, cADPR caused an increase in the frequency of Ca^{2+} sparks in permeabilized myocytes.[14] Representative line scan images of a cell acquired before and at different times after addition of 5 μmol/L cADPR are shown in Figure 1A. The changes in frequency and amplitude of events along with the values of basal fluorescence in the same experiment are documented in panel B. After a fast (≈0.1-second) application of the agent, the frequency of events gradually (ie, within 2 to 5 minutes) increased from approximately 4 to 8 events/s/100 μm and, in experiments without washout, remained elevated at this level for the duration of the experiment (10 minutes, n=5, not shown). The basal fluorescence was relatively constant (gray bars), suggesting that laser illumination did not cause significant dye bleaching or photo-damage to the cell in the course of the experiment. The effect of cADPR on Ca^{2+} sparks was reversible, as the frequency of events returned to levels close to control within 2 to 3 minutes after the removal of cADPR. The results of the effects of cADPR on Ca^{2+} sparks are summarized in Figure 1C. As can be seen, cADPR caused approximately a 50% increase (47±9%, $P<0.001$) in the frequency of detected events. At the same time, although there was no significant change in the average amplitude of events, the magnitude of the 5% of the brightest sparks (most likely to be situated at the center of the line scan) significantly increased in each of the consecutive images (22±10%, $P<0.05$, n=5). Consistent with our previous observations,[14] cADPR had no significant effects on the half-maximal duration (21.0±1.1 versus 20.8±1.0 ms in control and presence of 5 μmol/L cADPR, respectively) and half-maximal width (1.9±0.1 and 1.6±0.2 μm in control and presence of 5 μmol/L cADPR, respectively) of the events. The potentiation of Ca^{2+} release by cADPR was prevented by the specific antagonist of cADPR 8-Br-cADPR,[19] confirming that the effects of cADPR were on specific cADPR binding sites (Figure 2). In this series of experiments, the cardiomyocytes were pretreated with 5 μmol/L 8-Br-cADPR for 5 minutes before the exposure to cADPR. Under these conditions, cADPR failed to produce any changes in the frequency and amplitude of events (Figure 2B). Taken together, these results are consistent with the ability of cADPR to produce a slow enhancement in the functional activity of Ca^{2+} release sites by activating a cADPR-specific signaling pathway.

Effects of Direct Activation of RyRs on Ca^{2+} Sparks

To better understand the mechanism of action of cADPR on Ca^{2+} release, we contrasted the impact of cADPR with that of the well-known RyR agonist caffeine on spontaneous Ca^{2+} sparks in permeabilized myocytes. We used small concentrations of caffeine (0.1 to 0.3 mmol/L) to produce potentiating effects of similar scale as observed with cADPR. In contrast to the slow and maintained effects observed with cADPR, caffeine produced rapid and transient enhancement in the frequency of events (by ≈60%).





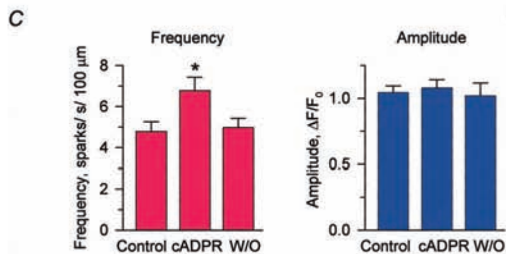


Figure 1. Effects of cADPR on properties of Ca^{2+} sparks in permeabilized rat ventricular myocytes. A, Representative line scan fluorescence images under control conditions (a), and at different times (b through d) after exposure of the cell to 5 μmol/L cADPR (the experimental protocol is presented schematically at the top). B, Ca^{2+} spark frequency (dark red) and amplitude (blue), and F_0 from corresponding images as a function of time before and after the addition of cADPR (arrow) into the bathing solution in the same experiment. The basal fluorescence is indicated by the gray portion at the bottom of the amplitude bars. Stages a through d correspond to images depicted by panel A. C, Averaged spark frequency (red) and amplitude (blue) in control solution 5 minutes after application of cADPR and 3 minutes after washout of the drug. $^*P<0.01$, $n=10$.

These were followed by a decrease in sparking activity below the control level. The effects of 0.1 mmol/L caffeine on frequency and amplitude of Ca^{2+} sparks are illustrated in Figure 3. Similar rapid and transient changes in spark frequency on the addition of small doses of caffeine were observed in 10 cells used in this series of experiments. The rapid action of caffeine is consistent with the direct interaction of this agent with the RyRs. The secondary decline in frequency of events could be caused by reductions in the SR Ca^{2+} content as a result of enhanced Ca^{2+} release through RyRs. The profound differences in the action of cADPR and caffeine on Ca^{2+} sparks suggest that cADPR acts through a different mechanism, specifically one that does not involve a direct activation of the RyR.

Effects of cADPR on SR Ca^{2+} Content

To further explore the mechanisms of action of cADPR, we performed measurements of the SR Ca^{2+} content using large concentrations of caffeine (20 mmol/L) to liberate Ca^{2+} from the SR. Exposure of the cells to 5 μmol/L cADPR for 5 minutes caused approximately a 50% increase in the peak amplitude of caffeine-induced Ca^{2+} transients (from 3.2 ± 0.5 to 4.7 ± 0.4 $\Delta F/F_0$, $P<0.01$; Figure 4A and 4C), implying that the SR Ca^{2+} load was enhanced by cADPR. Under similar conditions, exposure of the cells to 0.1 mmol/L caffeine, the amplitude of Ca^{2+} transients decreased by $\approx$46% (from 3.1 ± 0.4 to 1.6 ± 0.4 $\Delta F/F_0$, $P<0.05$; Figure 4B and 4D), indicating a reduction in the SR Ca^{2+} load. This effect of caffeine is consistent with a direct and selective activation of

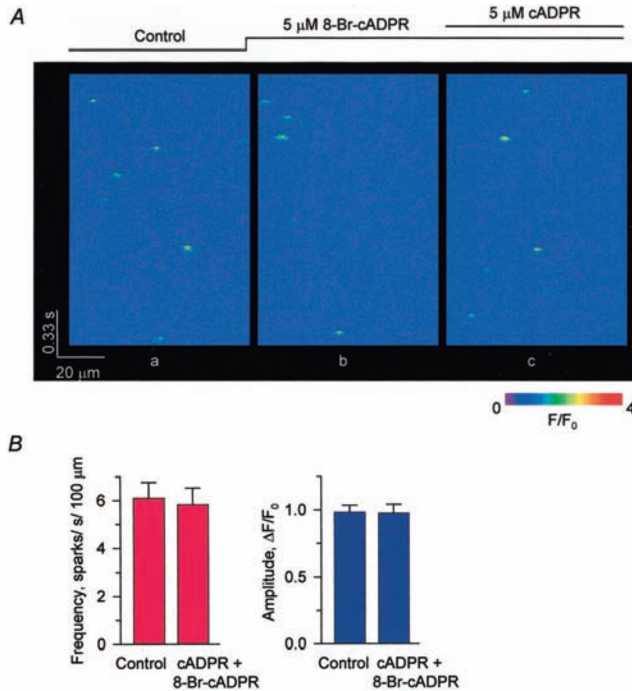


Figure 2. Prevention of cADPR-induced potentiation of Ca^{2+} sparks by 8-Br-cADPR. A, Representative line scan fluorescence images under control conditions (a), 1 minute after exposure of the cell to 5 μmol/L 8-Br-cADPR (b), and 5 minutes after 5 μmol/L of cADPR was added into the bathing solution (c). The experimental protocol is presented schematically at the top. B, Averaged spark frequency (red) and amplitude (blue) in control solution and in 5 minutes after application of 5 μmol/L cADPR (n=5).

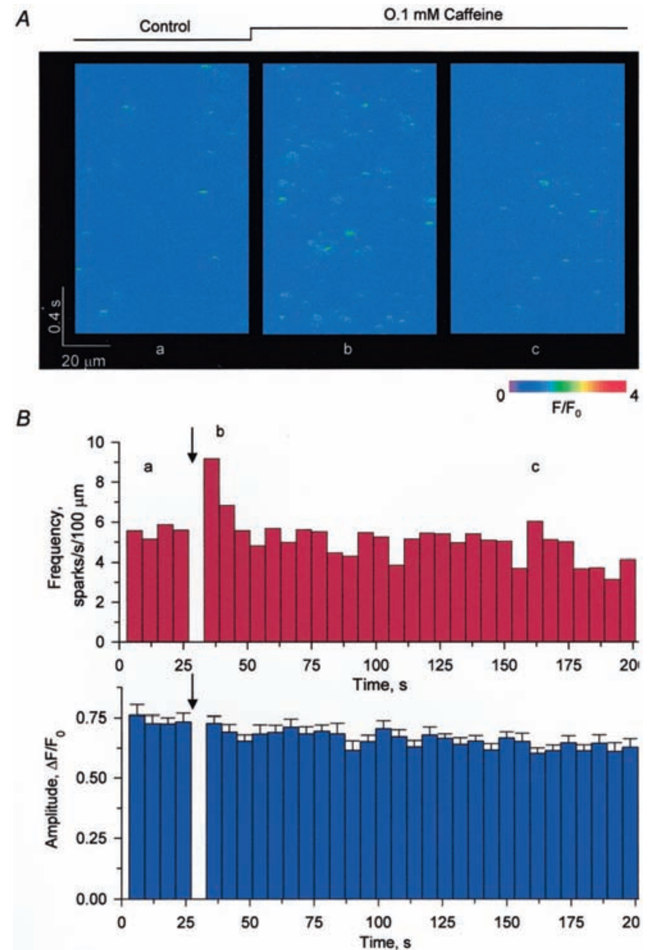


Figure 3. Effects of low doses of caffeine on properties of Ca^{2+} sparks. A, Representative line scan fluorescence images under control conditions (a), as well as 1 ssecond and 2.5 minutes after exposure of the cell to 0.1 mmol/L caffeine (b and c, respectively). The experimental protocol is presented schematically at the top. B, Ca^{2+} spark frequency (dark red) and amplitude (gray) as a function of time before and after the addition of caffeine (arrow) into the bathing solution in the same experiment.

RyRs, which leads to a net loss in the SR Ca^{2+} content. On the other hand, the ability of cADPR to increase the SR Ca^{2+} content suggests that its effects on SR Ca^{2+} release might be mediated by potentiating the uptake of Ca^{2+} into the SR. Elevation of SR Ca^{2+} content has been reported to increase the frequencies of Ca^{2+} sparks in both intact and permeabilized cardiac myocytes.[17,20,21] Thus, the enhanced sparking activity illustrated in Figure 1 could be secondary to elevation of luminal Ca^{2+} in the presence of cADPR.

Preventing Effects of Thapsigargin

To test whether changes in the SR Ca^{2+} content in fact are necessary and sufficient to produce the cADPR-induced increase in sparking activity, we used a specific inhibitor of the SR Ca^{2+} pump (SERCA), thapsigargin, in concert with cADPR. If enhanced accumulation of Ca^{2+} were required for potentiation of Ca^{2+} sparks by cADPR, one would expect little potentiation of Ca^{2+} sparks when the resequestration mechanism is suppressed by thapsigargin. This hypothesis was supported by the results of our experiments (Figure 5). In this series of experiments, the cardiomyocytes were pretreated with 0.1 mmol/L thapsigargin for 1 minute before addition of cADPR. As shown previously,[22] thapsigargin at this concentration reduces SR Ca^{2+} accumulation without altering the probability of generation of Ca^{2+} sparks at resting cytosolic [Ca^{2+}] (≈100 nmol/L). With this protocol, the SR Ca^{2+} content of cells exposed to thapsigargin and cADPR was not significantly different from that of control cells (−5±4.3%, n=6; Figure 5D). As illustrated by panels A through C of Figure 5, when the SR Ca^{2+} load of cells exposed to cADPR was the same as it was in control cells, cADPR failed to induce any changes in Ca^{2+} sparks. In several cells, we also examined the effects of thapsigargin alone on Ca^{2+} sparks. In accordance with Song et al,[22] 100 nmol/L thapsigargin had no significant effects on spontaneous activity of release sites (the frequency of sparks was 4.8±0.8 and 4.1±7 events/s/100 μm in control and 5 minutes after addition of the drug, respectively, n=5). This apparent lack of influence of reduced SR Ca^{2+} content on release sites can be attributed to the relatively low affinity and steep dependence of the RyR on luminal Ca^{2+} (K_D≈2.5 mmol/L; Hill Coefficient≈2).[18] Shannon and Bers[23] have estimated that the resting [Ca^{2+}] inside the SR approaches only about 0.7 mmol/L at cytosolic [Ca^{2+}] of 100 nmol/L. Therefore, reducing SR Ca^{2+} accumulation may simply decrease $[Ca^{2+}]_{SR}$ below the range where it can effectively influence

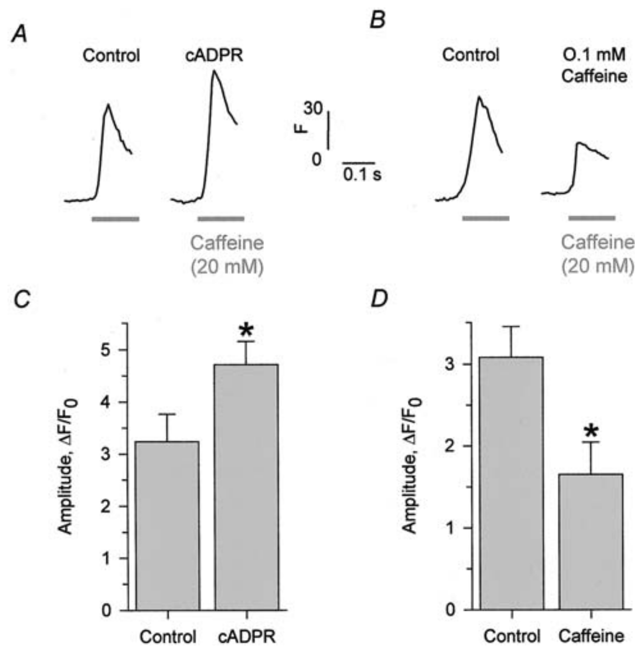


Figure 4. Effects of cADPR and caffeine on SR Ca^{2+} content. A and B, Representative Ca^{2+} transients induced by application of 20 mmol/L caffeine to the whole bath under control conditions and in the presence of 5 μmol/L cADPR (A) or 0.1 mmol/L caffeine (B). C and D, Averaged amplitude of caffeine-induced Ca^{2+} transients in the control solution and 5 minutes after application of cADPR (C, n=5) or 0.1 mmol/L caffeine (D, n=5).

RyR gating. Taken together, these results suggest that the increase in frequency of Ca^{2+} sparks by cADPR is caused not by direct sensitization of the RyR, but rather it is mediated by elevation in the SR Ca^{2+} content.

Lipid Bilayer Experiments

To evaluate the ability of cADPR to influence the functional activity of the RyR more directly, we performed experiments with single RyRs reconstituted into lipid bilayers. Heavy cardiac SR microsomes were fused into planar lipid bilayers, and single RyR channels were monitored by using Cs^+ as the charge carrier. The solutions on the cytosolic (*cis*) side of the channel contained 3 mmol/L ATP and 3.6 mmol/L Mg^{2+} ($[Mg^{2+}]_{free}$=≈0.9 mmol/L) to match the concentration of these ions in the internal solutions in our permeabilized myocyte experiments. Addition of cADPR (5 μmol/L) to the cytosolic side of the channel had no significant effect on the open probability (P_o) of the channel (Figure 6). Our results are in agreement with previous studies reporting no significant effects of cADPR on the RyR in the presence of physiological [ATP].[7] They are also consistent with the results of our experiments in permeabilized myocytes (see above), suggesting that the effects of cADPR on RyR involve indirect mechanisms, such as activation by elevated luminal Ca^{2+}.

SR Ca^{2+} Uptake Measurements

To examine the effects of cADPR on Ca^{2+} uptake by the SERCA, we performed Ca^{2+} uptake measurements fluorometrically in isolated cardiac microsomes using Fura 2FF. To inhibit the Ca^{2+} leak through the RyRs, the medium was supplemented with 10 μmol/L ruthenium red, a known RyR channel blocker. Active Ca^{2+} uptake was initiated by administration to the cuvette solution of 500 nmol/L Ca^{2+}. Thus, the preparation featured simple uptake to the SR by SERCA without concurrent release via RyRs. In the representative experiment shown in Figure 7A, free calcium concentration outside of the microsomes declined much more rapidly in the presence of cADPR. The decay constant in the presence of the modulator was approximately half that of the control. Based on results of 17 independent measurements like that in 7A, we found the mean decay constant in the presence of cADPR to be approximately 20 seconds. This compares to an average of 40 seconds for control conditions. This confirms the results of our experiments in permeabilized cells, suggesting that cADPR enhances the rate of Ca^{2+} uptake by the SR and establishes the SR Ca^{2+} uptake mechanism as a likely target of cADPR in the cardiac muscle.

Discussion

cADPR is commonly viewed as a specific agonist of RyR channels that acts by sensitizing the RyR to cytosolic Ca^{2+}. Our results show for the first time that the primary target of cADPR in the heart is SERCA-dependent Ca^{2+} uptake. Potentiation of Ca^{2+} release by cADPR is mediated by increased SR Ca^{2+} load and subsequent luminal Ca^{2+}–dependent activation of RyRs (Figure 8). Luminal Ca^{2+} is increasingly recognized as a modulator of RyR activity in cardiac muscle. It has been recently shown that increases in $[Ca^{2+}]_{SR}$ are detected at specific sites on the luminal side of the RyR or associated regulatory protein. Elevated luminal calcium enhances the open probability of the channel.[18,24] The luminal sensor mechanism, which links the functional activity of the RyR to the loading state of the SR, appears to be a part of a dynamic control system that allows the cells to autoregulate the size and functional state of their SR Ca^{2+} pool.[17,18,25] In the present study, we show that activation of RyRs by Ca^{2+} within the SR also represents the final downstream step in a cADPR-dependent signaling cascade that operates to produce maintained positive inotropic effects on the heart.

Our finding that cADPR acts by enhancing the SR Ca^{2+} uptake reconciles a body of apparently contradictory results. First, it helps to explain why cADPR has no significant effect on isolated RyR channels reconstituted into lipid bilayers[6,7] (and the present study), although it enhances SR Ca^{2+} release from ryanodine-sensitive stores in most in situ studies.[10–12,14] Several previous studies did report increases in RyR P_o under simple ionic conditions (ie, absence of ATP).[5,26] Those effects, however, can be attributed to the action of cADPR on adenine nucleotide binding sites.[7] This explains why cADPR appears to be ineffective in the presence of millimolar ATP. Second, our results could also account for the slow time course and high temperature dependence of the effects of cADPR described in the literature.[10,11] The slow time course can be ascribed to the dynamic lag associated with accumulation of Ca^{2+} in the SR, a lag which would not be present if cADPR acted directly on the RyR. The high temperature dependence is consistent with the fact that the SR Ca^{2+}ATPase is more temperature dependent than the RyR (the former has a Q_{10}=2.2, whereas the latter has a Q_{10}=1.5).[27] Based on the reported temperature dependence of

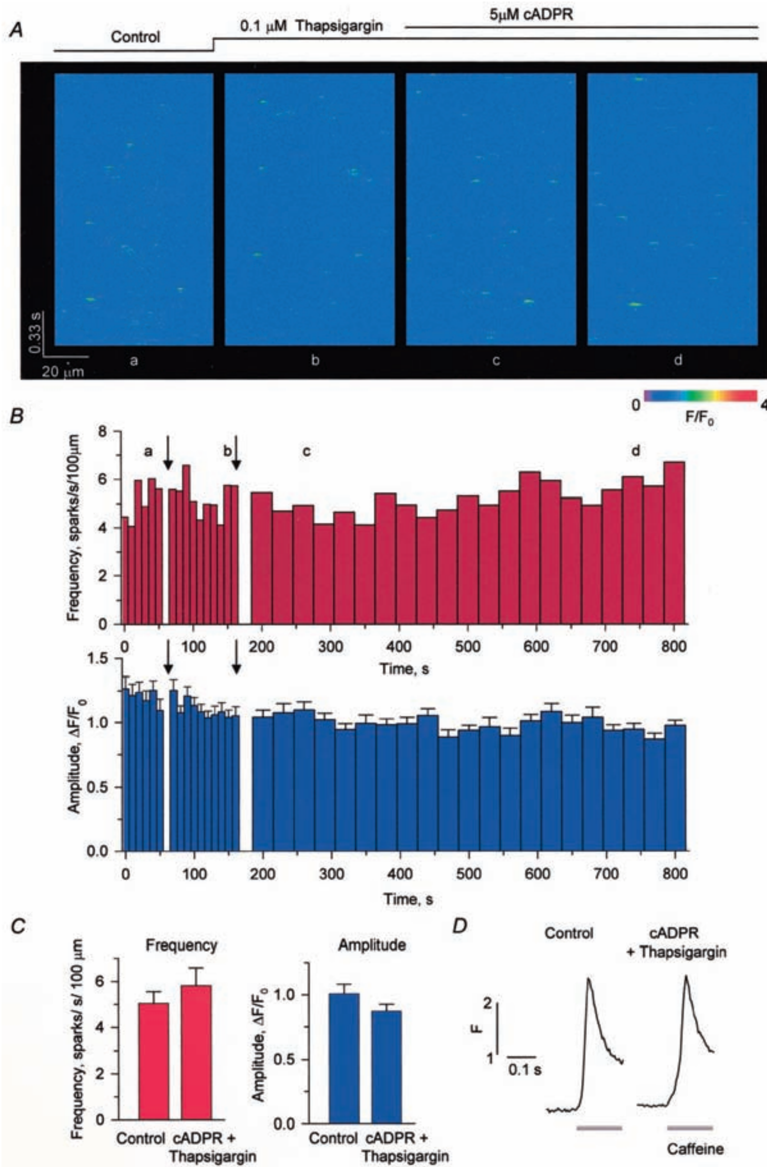


Figure 5. Preventing cADPR-induced potentiation of Ca^{2+} sparks by thapsigargin. A, Representative line scan fluorescence images under control conditions (a), 1 minute after addition of 0.1 μmol/L thapsigargin (b) and at different times (c and d) after administration of 5 μmol/L cADPR (the experimental protocol is presented schematically at the top panel). B, Ca^{2+} spark frequency (dark red) and amplitude (blue) as a function of time before and after the addition of thapsigargin and cADPR into the bathing solution in the same experiment. Stages a through d correspond to images represented in the panel A. C, averaged spark frequency (red) and amplitude (blue) in control and 5 minutes after application of cADPR in the presence of 0.1 μmol/L thapsigargin (n=8). D, representative Ca^{2+} transients induced by application of 20 mmol/L caffeine to the whole bath under control conditions and in the presence of cADPR and thapsigargin (5 minutes).

the effects of cADPR on whole-cell Ca^{2+} transients,[10] we would expect the effects observed in this study at room temperature to be even more pronounced at body temperature. Finally, our findings explain how cADPR produces maintained stimulation of RyR activity without causing a compensatory decrease in the loading of the SR by Ca^{2+}. It has been shown previously and confirmed here (Figure 3) that specific modulators of RyR activity, such as the agonist caffeine and the inhibitor tetracaine produce only transient effects on Ca^{2+} sparks in resting cardiac myocytes.[15,17] The transient nature of the effects arises as a result of SR Ca^{2+} load compensating for the primary changes in availability of the RyR with subsequent luminal Ca^{2+}–dependent changes in release site activity. Similar biphasic effects of tetracaine and caffeine on SR Ca^{2+} release have been observed with globally measured systolic Ca^{2+} transients in rat ventricular myocytes.[25,28] One logical consequence of this dynamic regulation process is that maintained potentiation of SR Ca^{2+} release can be attained only by enhancing Ca^{2+} uptake or when the Ca^{2+} uptake and the release mechanisms are stimulated simultaneously.[25] Adrenergic stimulation of the SERCA and the RyR can be regarded as a well-established example of the second scenario. The effect of cADPR appears to present a case of a more specific modulatory influence, in which the release is enhanced solely by increasing the uptake, with no direct effects on the RyRs.

Although affecting the frequency of sparks, cADPR did not significantly influence their spatio-temporal characteristics. If the SERCA pump function is altered by cADPR, one might also expect changes in these parameters.[29] The decline of $[Ca^{2+}]$ during the Ca^{2+} sparks is determined primarily by Ca^{2+} diffusion from the source of Ca^{2+}.[30] Indeed, complete inhibition of the SERCA pump by 5 μmol/L thapsigargin has been shown to cause only a relatively small prolongation of the $[Ca^{2+}]$ decay (by $\approx$30%) during Ca^{2+} sparks.[30] Thus, the cADPR-mediated change in the pump rate might not be sufficiently large to manifest itself in altered spark decay kinetics. In support of this explanation, 100 nmol/L thapsi-

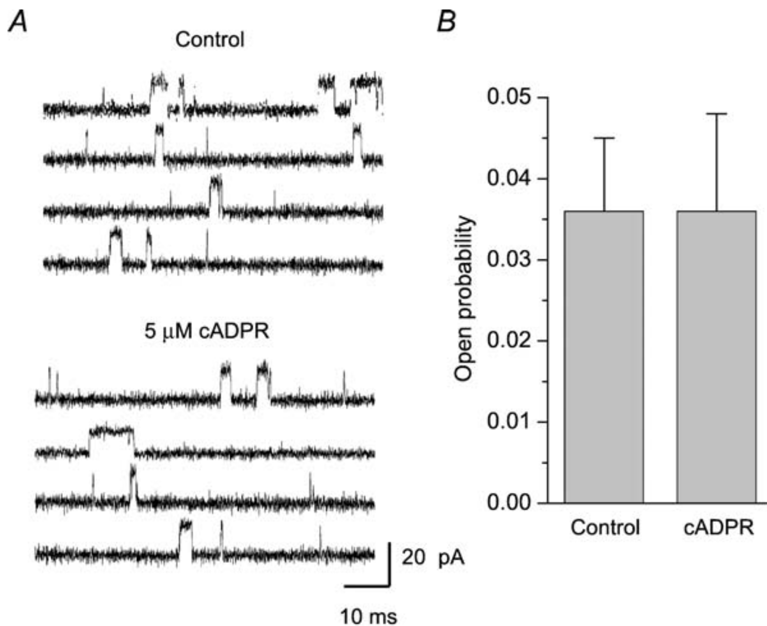


Figure 6. Effect of cADPR on characteristics of single ryanodine receptor channels. A, Representative single channel recordings performed in symmetrical Cs^+ and Ca^{2+} under control conditions and after addition (4 minutes) of 5 μmol/L cADPR. The *cis* chamber contained 3 mmol/L MgATP and 0.6 mmol/L $MgCl_2$ ($[Mg^{2+}]_{free}$=≈0.9 mmol/L). Channel openings are upward. Holding potential was +30 mV. B, Averaged (means±SEM) P_o in the control and presence of cADPR ($P>0.5$; n=4).

gargin selected to prevent the enhancement of the SR Ca^{2+} load by cADPR produced no significant changes in the spatio-temporal properties of sparks (Figure 3A). Consistent with our results, Santana et al[31] found no significant difference in the decay time constants of sparks measured in ventricular myocytes from wild type and phospholamban-deficient transgenic mice, although the SR Ca^{2+} load and the frequency of events were significantly increased in transgenic cells.

It has been reported that the decrease of the depolarization-induced Ca^{2+} transients and contractions produced by the cADPR antagonist 8-amino-cADPR is not accompanied by any decrease of SR Ca^{2+} content assessed by caffeine-induced contractions.[8] This would imply that the effects of cADPR and its antagonists are not mediated by changes in the SR Ca^{2+} content. The reasons for these discrepancies are not known, but they may involve differences in experimental approaches. Myofilament Ca^{2+} sensitivity is subject to modulation by various factors, which might complicate the interpretation of results obtained with contraction measurements. For example, methylxanthines, including caffeine, are potent myofilament Ca^2 sensitizers that can influence contractile performance either through direct interaction with contractile proteins or via their ability to inhibit cardiac phosphodiesterases and thereby elevate cAMP.[1] Thus, it is possible that the estimates of SR Ca^{2+} content changes using contraction measurements were influenced by effects of caffeine on myofilaments. Interestingly, in a more recent study, Rakovic et al[9] showed that 8-amino-cADPR reduced the frequency of spontaneous Ca^{2+} waves, although cADPR itself enhanced the incidents of waves in cardiac myocytes exposed to ouabain. Spontaneous Ca^{2+} waves are generally associated with increased SR Ca^{2+} content. RyR modulators such as the inhibitor tetracaine and the potentiator caffeine have been shown to cause only transient effects on Ca^{2+} waves because the alterations of RyR activity are compensated by changes in load.[15,25,28] Therefore, the sustained effects of cADPR and its antagonists on Ca^{2+} waves are more likely to be the result of SR pump-mediated changes in SR Ca^{2+} load rather than to modulation of the RyRs.

At the present time, we do not know the specific biochemical mechanisms underlying the enhancement of SR Ca^{2+} uptake by cADPR. Theoretically, increased SR Ca^{2+} uptake could be the result of either inhibition of Ca^{2+} leak or stimulation of Ca^{2+} uptake by the SERCA. It has been shown previously that RyRs represent the single most prominent

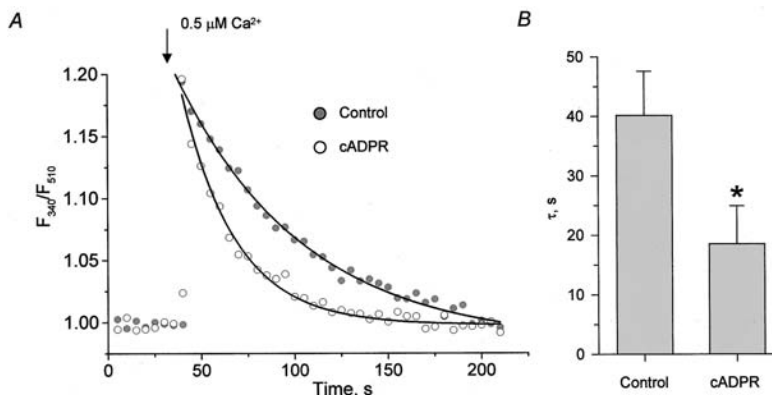


Figure 7. Effects of cADPR on Ca^{2+} uptake by cardiac SR vesicles. A, Cardiac microsomes (0.6 mg/mL) were administered 500 nmol/L $CaCl_2$ under control conditions (●) and in the presence of 5 μmol/L cADPR (○), both in the presence of 10 μmol/L ruthenium red. The time courses of uptake were best fit by exponential functions (solid lines). B, Averaged (means±SEM) time constants of Ca^{2+} uptake in the control and presence of cADPR ($P<0.05$; 10 and 8 independent measurements, respectively).

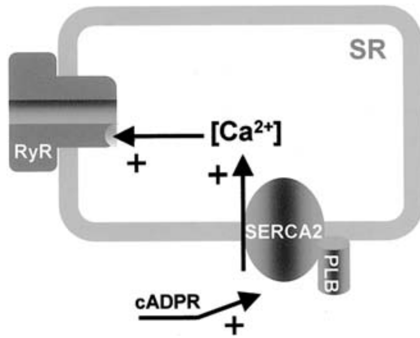


Figure 8. Schematic presentation of the mechanism of action of cADPR on SR Ca^{2+} release in cardiac myocytes. cADPR enhances SR Ca^{2+} uptake either through direct activation of the SERCA pump or through relieving the inhibition of the pump by phospholamban (PLB). This results in elevation of $[Ca^{2+}]$ in the lumen of the SR. The increased $[Ca^{2+}]_{SR}$ enhances the functional activity of the RyR through luminal Ca^{2+} sensing sites.

pathway of Ca^{2+} leak from the SR in cardiac myocytes.[32,33] In our SR Ca^{2+} uptake measurements, the RyRs were inhibited by ruthenium red. In addition, cADPR has been reported to have no inhibitory effects on the RyR channels in vitro. Therefore it is unlikely that the increased rate of net Ca^{2+} uptake in our experiments is caused by reduced SR Ca^{2+} leak. Stimulation of the SERCA by cADPR could be produced either by direct potentiation of the SERCA or through the relieving of the inhibition of dephosphorylated phospholamban. Indeed, several agents have been reported either to stimulate the SERCA, such as gingerol and 1-(3,4-dimethoxyphenyl)-3-dodeconone,[34,35] or to inhibit the phospholamban interactions as described with tannin, ellagic acid and polyanionic compounds.[35,36] Phospholamban is expressed primarily in the cardiac, smooth, and slow-twitch skeletal muscles, and it has been found only in mammalian and avian species.[37] On the other hand, the intracellular Ca^{2+} mobilizing effects of cADPR have been described in various cell types across different phyla. This lack of correlation between the effects of cADPR and the presence of phospholamban would seem to imply that stimulation of Ca^{2+} uptake occurs through a mechanism that does not require the involvement of phospholamban. It is possible however that in other cellular systems, the role of phospholamban is played by homologous regulatory proteins. Regardless of the specific biochemical mechanisms, stimulation of SR Ca^{2+} uptake plays an important role in the enhancement of Ca^{2+} release through RyRs in cardiac muscle. Future studies will have to determine precisely how cADPR causes stimulation of SR Ca^{2+} uptake in the heart and whether these mechanisms apply to other cell types.

Acknowledgments

This work was supported by grants from the American Heart Association, Texas Affiliate (V.L.) and the National Institutes of Health (HL 52620 and HL 03739 to S.G.).

References

1. Bers DM. *Excitation-Contraction Coupling, and Cardiac Contractile Force*. Dordrecht, the Netherlands: Kluwer Academic Publishers; 1991.
2. Wier WG, Balke CW. Ca^{2+} release mechanisms, Ca^{2+} sparks, and local control of excitation-contraction coupling in normal heart muscle. *Circ Res*. 1999;85:770–776.
3. Coronado R, Morrissette J, Sukhareva M, Vaughan DM. Structure and function of ryanodine receptors. *Am J Physiol*. 1994;266: C1485–C1504.
4. Lee HC. Physiological functions of cyclic ADP-ribose and NAADP as calcium messengers. *Annu Rev Pharmacol Toxicol*. 2001;41:317–345.
5. Meszaros LG, Bak J, Chu A. Cyclic ADP-ribose as an endogenous regulator of the non-skeletal type ryanodine receptor Ca^{2+} channel. *Nature*. 1993;364:76–79.
6. Fruen BR, Mickelson JR, Shomer NH, Velez P, Louis CF. Cyclic ADP-ribose does not affect cardiac or skeletal muscle ryanodine receptors. *FEBS Lett*. 1994;352:123–126.
7. Sitsapesan R, McGarry SJ, Williams AJ. Cyclic ADP-ribose competes with ATP for the adenine nucleotide binding site on the cardiac ryanodine receptor Ca^{2+}-release channel. *Circ Res*. 1994;75:596–600.
8. Rakovic S, Galione A, Ashamu GA, Potter BV, Terrar DA. A specific cyclic ADP-ribose antagonist inhibits cardiac excitation-contraction coupling. *Curr Biol*. 1996;6:989–996.
9. Rakovic S, Cui Y, Iino S, Galione A, Ashamu GA, Potter BVL, Terrar DA. An antagonist of cADP-ribose inhibits arrhythmogenic oscillations of intracellular Ca^{2+} in heart cells. *J Biol Chem*. 1999;274: 17820–17827.
10. Iino S, Cui Y, Galione A, Terrar DA. Actions of cADP-ribose and its antagonists on contraction in guinea pig isolated ventricular myocytes: influence of temperature. *Circ Res*. 1997;81:879–884.
11. Cui Y, Galione A, Terrar DA. Effects of photoreleased cADP-ribose on calcium transients and calcium sparks in myocytes isolated from guinea-pig and rat ventricle. *Biochem J*. 1999;342:269–273.
12. Prakash YS, Kannan MS, Walseth TF, Sieck GC. ADP ribose and $[Ca^{2+}]_i$ regulation in rat cardiac myocytes. *Am J Physiol*. 2000;279: H1482–H1489.
13. Guo X, Laflamme MA, Becker PL. Cyclic ADP-ribose does not regulate sarcoplasmic reticulum Ca^{2+} release in intact cardiac myocytes. *Circ Res*. 1996;79:147–151.
14. Lukyanenko V, Györke S. Ca^{2+} sparks and Ca^{2+} waves in saponin-permeabilized cardiac myocytes. *J Physiol (Lond)*. 1999;521:575–585.
15. Györke S, Lukyanenko V, Györke I. Dual effects of tetracaine on spontaneous calcium release in rat ventricular myocytes. *J Physiol (Lond)*. 1997;500:2297–2309.
16. Lukyanenko V, Györke I, Subramanian S, Smirnov A, Wiesner TF, Györke S. Inhibition of Ca^{2+} sparks by ruthenium red in permeabilized rat ventricular myocytes. *Biophys J*. 2000;79:1273–1284.
17. Lukyanenko V, Viatchenko-Karpinski S, Smirnov A, Wiesner TF, Györke S. Dynamic regulation of the SR Ca^{2+} content and release by luminal Ca^{2+}-sensitive leak through RyRs in rat ventricular myocytes. *Biophys J*. 2001;81:785–798.
18. Györke I, Györke S. Regulation of the cardiac ryanodine receptor channel by luminal Ca^{2+} involves luminal Ca^{2+} sensing sites. *Biophys J*. 1998; 75:2801–2810.
19. Walseth TF, Lee HC. Synthesis and characterization of antagonists of cyclic-ADP-ribose–induced Ca^{2+} release. *Biochim Biophys Acta*. 1993; 1178:235–242.
20. Cheng H, Lederer MR, Lederer WJ, Cannell MB. Calcium sparks and $[Ca^{2+}]_i$ waves in cardiac myocytes. *Am J Physiol*. 1996;270:C148–C159.
21. Lukyanenko V, Györke I, Györke S. Regulation of calcium release by calcium inside the sarcoplasmic reticulum in ventricular myocytes. *Pflugers Arch*. 1996;432:1047–1054.
22. Song LS, Stern MD, Lakatta EG, Cheng H. Partial depletion of sarcoplasmic reticulum calcium does not prevent calcium sparks in rat ventricular myocytes. *J Physiol*. 1997;505:665–675.
23. Shannon TR, Bers DM. Assessment of intra-SR free [Ca] and buffering in rat heart. *Biophys J*. 1997;73:1524–1531.
24. Ching LL, Williams AJ, Sitsapesan R. Evidence for Ca^{2+} activation and inactivation sites on the luminal side of the cardiac ryanodine receptor complex. *Circ Res*. 2000;87:201–206.
25. Eisner DA, Trafford AW, Diaz ME, Overend CL, O'Neill SC. The control of Ca release from the cardiac sarcoplasmic reticulum: regulation versus autoregulation. *Cardiovasc Res*. 1998;38:589–604.
26. Li PL, Tang WX, Valdivia HH, Zou AP, Campbell WB. cADP-ribose activates reconstituted ryanodine receptors from coronary arterial smooth muscle. *Am J Physiol*. 2001;280:H208–H215.
27. Sitsapesan R, Montgomery RA, MacLeod KT, Williams AJ. Sheep cardiac sarcoplasmic reticulum calcium-release channels: modifi-

cation of conductance and gating by temperature. *J Physiol*. 1991;434: 469–488.
28. Eisner DA, Choi HS, Diaz ME, O'Neill SC, Trafford AW. Integrative analysis of calcium cycling in cardiac muscle. *Circ Res*. 2000;87: 1087–1094.
29. Huser J, Bers DM, Blatter LA. Subcellular properties of $[Ca^{2+}]_i$ transients in phospholamban-deficient mouse ventricular cells. *Am J Physiol*. 1998; 274:H1800–H1811.
30. Gomez AM, Cheng H, Lederer WJ, Bers DM. Ca^{2+} diffusion and sarcoplasmic reticulum transport both contribute to $[Ca^{2+}]_i$ decline during Ca^{2+} sparks in rat ventricular myocytes. *J Physiol*. 1996;496: 575–581.
31. Santana LF, Kranias EG, Lederer WJ. Calcium sparks and excitation-contraction coupling in phospholamban-deficient mouse ventricular myocytes. *J Physiol*. 1997;503:21–29.
32. Deleted in proof.
33. Bassani RA, Bers DM. Rate of diastolic Ca release from the sarcoplasmic reticulum of intact rabbit and rat ventricular myocytes. *Biophys J*. 1995; 68:2015–2022.
34. Kobayashi M, Shoji N, Ohizumi Y. Gingerol, a novel cardiotonic agent, activates the Ca^{2+}-pumping ATPase in skeletal and cardiac sarcoplasmic reticulum. *Biochim Biophys Acta*. 1987;903:196–102.
35. Berrebi-Bertrand I, Lahouratate P, Lahouratate V, Camelin JC, Guibert J, Bril A. Mechanism of action of sarcoplasmic reticulum calcium-uptake activators: discrimination between sarco(endo)plasmic reticulum Ca^{2+} ATPase and phospholamban interaction. *Eur J Biochem*. 1997;247:801–809.
36. Xu ZC, Kirchberger MA. Modulation by polyelectrolytes of canine cardiac microsomal calcium uptake and the possible relationship to phospholamban. *J Biol Chem*. 1989;264:16644–16651.
37. Simmerman HK, Jones LR. Phospholamban, protein structure, mechanism of action, and role in cardiac function. *Physiol Rev*. 1998;78: L921–L947.