Luminal Ca\(^{2+}\) Controls Termination and Refractory Behavior of Ca\(^{2+}\)-Induced Ca\(^{2+}\) Release in Cardiac Myocytes

Dmitry Terentyev, Sergei Viatchenko-Karpinski, Hector H. Valdivia, Ariel L. Escobar, Sandor Györke

Abstract—Despite extensive research, the mechanisms responsible for the graded nature and early termination of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the sarcoplasmic reticulum (SR) in cardiac muscle remain poorly understood. Suggested mechanisms include cytosolic Ca\(^{2+}\)-dependent inactivation/adaptation and luminal Ca\(^{2+}\)-dependent deactivation of the SR Ca\(^{2+}\) release channels/ryanodine receptors (RyRs). To explore the importance of cytosolic versus luminal Ca\(^{2+}\) regulatory mechanisms in controlling CICR, we assessed the impact of intra-SR Ca\(^{2+}\) buffering on global and local Ca\(^{2+}\) release properties of patch-clamped or permeabilized rat ventricular myocytes. Exogenous, low-affinity Ca\(^{2+}\) buffers (5 to 20 mmol/L ADA, citrate or maleate) were introduced into the SR by exposing the cells to “internal” solutions containing the buffers. Enhanced Ca\(^{2+}\) buffering in the SR was confirmed by an increase in the total SR Ca\(^{2+}\) content, as revealed by application of caffeine. At the whole-cell level, intra-SR [Ca\(^{2+}\)] buffering dramatically increased the magnitude of Ca\(^{2+}\) transients induced by \(I_{\text{Ca}}\) and deranged the smoothly graded \(I_{\text{Ca}}\)-SR Ca\(^{2+}\) release relationship. The amplitude and time-to-peak of local Ca\(^{2+}\) release events, Ca\(^{2+}\) sparks, as well as the duration of local Ca\(^{2+}\) release fluxes underlying sparks were increased up to 2- to 3-fold. The exogenous Ca\(^{2+}\) buffers in the SR also reduced the frequency of repetitive activity observed at individual release sites in the presence of the RyR activator Imperatotoxin A. We conclude that regulation of RyR openings by local intra-SR [Ca\(^{2+}\)] is responsible for termination of CICR and for the subsequent restitution behavior of Ca\(^{2+}\) release sites in cardiac muscle. (Circ Res. 2002;91:414-420.)

Key Words: sarcoplasmic reticulum • excitation-contraction coupling • calcium-induced calcium release • ryanodine receptor

In cardiac muscle, the process of excitation-contraction (EC) coupling is mediated by Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels in the plasmalemma triggering Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) from the SR. This Ca\(^{2+}\) release is the result of spatial and temporal summation of brief and localized Ca\(^{2+}\) release events, i.e., “Ca\(^{2+}\) sparks” that can be visualized by fluorescence imaging. Each spark involves 6 to 30 individual Ca\(^{2+}\) release channels/ryanodine receptors (RyRs). The concerted openings of multiple RyRs appear to be due to cross-activation of channels by released Ca\(^{2+}\) and may also involve more direct interactions between neighboring channels, i.e., “coupled gating.” A fundamental question about CICR is how Ca\(^{2+}\) release is terminated. Once initiated, Ca\(^{2+}\) release from the regenerative clusters should continue until the Ca\(^{2+}\) stores are emptied. However, Ca\(^{2+}\) sparks feature abrupt termination. Such a robust termination of Ca\(^{2+}\) release is necessary for the relaxation of the cardiac muscle. Previous studies have indicated that termination of Ca\(^{2+}\) release cannot be simply accounted for by such passive extinguishing mechanisms as depletion of SR Ca\(^{2+}\) or stochastic closure of the release channels. Instead, it appears that release termination is more likely to involve changes in open probability of RyRs, either caused by Ca\(^{2+}\)-dependent inactivation/adaptation of RyRs or after changes in SR luminal [Ca\(^{2+}\)] (([Ca\(^{2+}\)]\text{SR}). The resting free [Ca\(^{2+}\)] in the SR has been estimated to be approximately 1 mmol/L. To some extent, [Ca\(^{2+}\)]\text{SR} appears to be buffered by low-affinity endogenous Ca\(^{2+}\) binding proteins, such as calsequestrin (K\(_D\) \approx 0.6 mmol/L), that are expressed in the SR lumen. If depletion of luminal Ca\(^{2+}\) was an important factor in termination of Ca\(^{2+}\) release, increasing luminal Ca\(^{2+}\) buffering would be expected to prolong Ca\(^{2+}\) release duration leading to enlarged and prolonged cytosolic Ca\(^{2+}\) transients. In disagreement with this scenario, overexpressing calsequestrin in mouse heart has been shown to result in marked reduction in cellular Ca\(^{2+}\) transients and contractions, although the total SR Ca\(^{2+}\) content (estimated by caffeine application) increased dramatically. Furthermore, the mice overexpressing calsequestrin developed cardiac hypertrophy and failure. Although overexpressed calsequestrin should clearly increase intra-SR [Ca\(^{2+}\)] buffering, the interpretation of the results of these studies is complicated by potential compensatory changes and direct effects of calsequestrin on RyR activity in transgenic myocytes.

Original received April 30, 2002; revision received July 25, 2002; accepted July 26, 2002.
From Texas Tech University HSC (D.T., S.V.-K., A.L.E., S.G.), Lubbock, Tex; and the University of Wisconsin (H.H.V.), Madison, Wis.
Correspondence to Sandor Györke, Dept of Physiology, Texas Tech University HSC, 3601 4th St, Lubbock, TX 79430. E-mail sandor.gyorke@ttuhsc.edu

© 2002 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org DOI: 10.1161/01.RES.0000032490.04207.BD
In the present study, we investigated the effects of low-affinity exogenous Ca\(^{2+}\) chelators loaded into the lumen of the SR on properties of global and local Ca\(^{2+}\) release in dialyzed patch-clamped and permeabilized rat ventricular myocytes. Inclusion of exogenous Ca\(^{2+}\) buffers into the SR should stabilize intra-SR [Ca\(^{2+}\)], thus enabling us to determine the relative importance of changes of [Ca\(^{2+}\)\(_{38}\)] in Ca\(^{2+}\) release termination. In addition, this strategy should allow us to probe the role of luminal Ca\(^{2+}\) buffers in the absence of potential nonspecific changes induced by protein overexpression in transgenic animals. We show that at variance with the results obtained in calsequestrin-overexpressing myocytes, enhanced luminal Ca\(^{2+}\) buffering increases the magnitude of Ca\(^{2+}\) release and at the same time slows the dynamics of functional recharging of SR Ca\(^{2+}\) stores. Based on our results, we conclude that termination of CICR and the subsequent restitution behavior of Ca\(^{2+}\) release sites in cardiac muscle are determined by changes in intra-SR [Ca\(^{2+}\)] regulating RyR openings.

Materials and Methods

Single ventricular myocytes were obtained from adult male Sprague-Dawley rat (Charles River Labs, Raleigh, NC) hearts by enzymatic dissociation.\(^{16}\) Rats were handled in accordance with the guidelines of the Texas Tech University HSC Animal Care and Use Committee. Experiments were performed either in patch-clamped or in permeabilized myocytes at room temperature (21 to 23°C). Whole-cell patch-clamp recordings of transmembrane currents were performed using an Axopatch 200B amplifier (Axon Instruments, USA). External solution contained (in mmol/L) 140 NaCl, 5.4 KCl, 1 CaCl\(_2\), 0.5 MgCl\(_2\), 10 HEPES, 5.6 glucose, and 0.02 TTX (pH 7.3). Micropipettes made from borosilicate glass (Sutter Instrument Co., USA; 1 to 3 MΩ resistance) were filled with a solution that contained (in mmol/L) 120 Cs\(-\)aspartate, 20 CsCl, 3 Na\(_2\)ATP, 3.5 MgCl\(_2\), 0.5 HEPES, 5.6 glucose, and 0.02 TTX (pH 7.3). Micropipettes containing 20 mmol/L citrate or maleate for 20 minutes were prepared by substituting Cs\(-\)aspartate with osmotically equivalent amounts of Cs-salts of the chelators. Free [Mg\(^{2+}\)]\(_{\text{free}}\) in the pipette solution was measured fluorometrically (with Mag-Fluo-4 [5 μmol/L], Molecular Probes) and adjusted to ~1 mmol/L. Voltage pulses were applied from a holding potential of −50 mV at 1-minute intervals, unless specified otherwise. Cardiac myocyte permeabilization was performed using saponin as described previously.\(^{16}\) The basic internal solution contained (in mmol/L) 100 K\(-\)aspartate, 20 KCl, 3 MgATP, 0.5 EGTA, 0.81 MgCl\(_2\), 10 phosphocreatine, 20 HEPES, 0.03 Fluo-3 K-salt (Te-fLabs), and 5 U/mL creatine phosphokinase, pCa 7 (pH 7.3). K-salts of citrate, maleate, acetamido iminodiacetic acid (ADA), and 5,5\'-dinitro-BAPTA (dn-BAPTA) were added into the internal solution at pCa 7 (pH 7.3). Pipette solutions containing various Ca\(^{2+}\) chelators (20 mmol/L of citrate, maleate or 5,5\'-dinitro-BAPTA) were prepared by substituting Cs\(-\)aspartate with osmotically equivalent amounts of Cs-salts of the chelators. Free [Mg\(^{2+}\)]\(_{\text{free}}\) in the pipette solution was measured fluorometrically (with Mag-Fluo-4 [5 μmol/L], Molecular Probes) and adjusted to ~1 mmol/L. Voltage pulses were applied from a holding potential of −50 mV at 1-minute intervals, unless specified otherwise. Cardiac myocyte permeabilization was performed using saponin as described previously.\(^{16}\) The basic internal solution contained (in mmol/L) 100 K\(-\)aspartate, 20 KCl, 3 MgATP, 0.5 EGTA, 0.81 MgCl\(_2\), 10 phosphocreatine, 20 HEPES, 0.03 Fluo-3 K-salt (Te-fLabs), and 5 U/mL creatine phosphokinase, pCa 7 (pH 7.3). K-salts of citrate, maleate, acetamido iminodiacetic acid (ADA), and 5,5\'-dinitro-BAPTA (dn-BAPTA) were added into the internal solution at pCa 7 (pH 7.3). K-salts of citrate, maleate, acetamido iminodiacetic acid (ADA), and 5,5\'-dinitro-BAPTA (dn-BAPTA) were added into the internal solution at specified concentrations by replacing osmotically equivalent amounts of K-aspartate. Free [Mg\(^{2+}\)]\(_{\text{free}}\) was adjusted to ~1 mmol/L in the solutions. Cells were imaged using a Bio-Rad Laser Scanning Confocal system (Bio-Rad MRC-1024ES interfaced to an Olympus IX-70 inverted microscope) with an Olympus 60×1.4 NA oil objective.\(^{16}\) Fluo-3 was excited by light at 488 nm, and the fluorescence was acquired at wavelengths of >515 nm in the line-scan mode of the confocal system at rate of 2.0 ms per scan. Ca\(^{2+}\) spark parameters were quantified using a detection/analysis computer algorithm.\(^{16}\) All chemicals unless specified otherwise were from Sigma. The local changes in cytosolic and luminal [Ca\(^{2+}\)] were simulated using a multicompartmental diffusion-reaction model of Ca\(^{2+}\) dynamics in the cytosolic and SR compartments. A detailed description of the model can be found in the online data supplement available at http://www.circresaha.org.

Results

Macroscopic Ca\(^{2+}\) Transients and I\(_{\text{Ca}}\) Measured Under Voltage Clamp

First, we explored the effects of dialysis of myocytes with internal solution containing the low-affinity Ca\(^{2+}\) chelators citrate and maleate (K\(_{\text{v}}\) values = 0.47 and 11 mmol/L, respectively)\(^{17}\) on EC coupling in patch-clamped ventricular myocytes. These anions may be effectively transported into the SR by an anion transporter, thus increasing the Ca\(^{2+}\) buffering capacity of the SR. Indeed, dialysis of the cells with solutions containing 20 mmol/L citrate or maleate for 20 minutes dramatically increased the amount of Ca\(^{2+}\) stored in the SR, as indicated by increased caffeine-induced Ca\(^{2+}\) transients (Figure 1A; see also online Table 2, which can be found in the online data supplement available at http://www.circresaha.org). This increase in the total SR Ca\(^{2+}\) content was associated with a marked enhancement of the amplitude of I\(_{\text{Ca}}\)-induced Ca\(^{2+}\) transients elicited by depolarization. In addition, the cells lost their ability to respond to I\(_{\text{Ca}}\) in a graded manner, because even
small Ca\(^{2+}\) currents triggered maximal Ca\(^{2+}\) transients (Figure 1B). Similar effects of intracellular citrate on Ca\(^{2+}\) transients have been observed previously in atrial myocytes.\(^{18}\) The increases in Ca\(^{2+}\) transients were more pronounced with citrate, which has a higher affinity for Ca\(^{2+}\) than with maleate. This is consistent with the notion that the effects of these Ca\(^{2+}\) chelators result from an increased Ca\(^{2+}\) storage capacity of the SR. Citrate should bind more Ca\(^{2+}\) ions in the SR lumen at free [Ca\(^{2+}\)] of \(\approx 1\) mmol/L, which is believed to correspond to the level of free [Ca\(^{2+}\)] in the SR at steady-state.\(^{2,12}\) These effects on Ca\(^{2+}\) transients were not accompanied by any significant changes in the amplitude of the Ca\(^{2+}\) currents, although \(I_{\text{Ca}}\) decay rate was slowed 1.4- to 2.7-fold, apparently due to interference of the buffers with Ca\(^{2+}\)-dependent inactivation (Figures 1A and 1B; online Table 2). Exposure of the myocytes to the high-molecular weight Ca\(^{2+}\) buffer dn-BAPTA (K\(_d\_\text{dn-BAPTA} = 7\) mmol/L) produced no significant changes in caffeine- and depolarization-induced Ca\(^{2+}\) transients, although it affected \(I_{\text{Ca}}\) inactivation in a manner similar to that observed with maleate and citrate (Figure 1B; online Table 2). These results may be due to inability of this compound to cross the SR membrane, a feature that would restrict its actions to the cytosolic compartment. These findings support the possibility that the bulk of the effects of maleate and citrate on Ca\(^{2+}\) transients was through the action of these compounds in the luminal rather than in the cytosolic compartment.

When citrate-dialyzed myocytes were stimulated periodically (at 0.5 Hz), they responded by alternating large and small Ca\(^{2+}\) transients. As illustrated by line-scan images (Figure 1C), the large-size Ca\(^{2+}\) transients were produced either by a massive and prolonged release of Ca\(^{2+}\), activated synchronously throughout the cell, or by a smaller, spatially inhomogeneous response eventually turning into full-scale regenerative Ca\(^{2+}\) release. Qualitatively, similar effects were observed in cells dialyzed with maleate but not dn-BAPTA, which had no significant impact on the periodic Ca\(^{2+}\) transients (not shown). In a separate series of 2-pulse experiments, intracellular dialysis with maleate or citrate also slowed the restitution of steady-state Ca\(^{2+}\) transients after Ca\(^{2+}\) release (online Figure 1, which can be found in the online data supplement available at http://www.circresaha.org). Taken together, these results suggest that exogenous Ca\(^{2+}\) buffers in the SR affect the processes of release and recharging of the SR Ca\(^{2+}\) stores by increasing the functional size of the stores. One significant limitation of the whole-cell dialysis experiments is that potential cytoplasmic effects of the chelators, such as effects on cytosolic Ca\(^{2+}\)-dependent inactivation of release, complicate interpretation of the results.

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

Therefore, to gain further mechanistic insights into the action of luminal Ca\(^{2+}\) buffers, we explored the effects of the same Ca\(^{2+}\) chelators as well as ADA (K\(_d\_\text{ADA} = 0.2\) mmol/L)\(^{7}\) on the Ca\(^{2+}\) storage capacity of the SR and Ca\(^{2+}\) release in permeabilized cardiac myocytes. Figure 2A shows traces of caffeine-induced Ca\(^{2+}\) transients in saponin-permeabilized myocytes that were incubated for various times with 20 mmol/L citrate or 5 mmol/L ADA before application of caffeine. Caffeine (10 mmol/L) was applied within 1 minute after reverting to the control, Ca\(^{2+}\)-chelator-free experimental solution. Exposure of the permeabilized myocytes to these Ca\(^{2+}\) chelators led to a gradual increase in the magnitude of the caffeine-induced Ca\(^{2+}\) transients, signal-

**Figure 2.** Effects of exposure to low-affinity Ca\(^{2+}\) chelators on properties of spontaneous Ca\(^{2+}\) sparks. The magnitude of events returned to control levels within 45 minutes in parallel with restoration of the total SR Ca\(^{2+}\) content.

**Figure 3.** Illustrates the effects of addition and subsequent removal from the internal solution of some of the aforementioned Ca\(^{2+}\) chelators on properties of spontaneous Ca\(^{2+}\) sparks. The brightness of the fluorescence events was reduced on addition of the chelators, apparently due to the action of buffers on the cytosolic side of the SR. However, removal of citrate and ADA from the internal solution after \(\approx 30\) minutes incubation of the myocytes with the chelators resulted in dramatic increases in the magnitude of Ca\(^{2+}\) sparks. The magnitude of events returned to control levels within 45 minutes in parallel with restoration of the total SR Ca\(^{2+}\) content (Figure 2B). We attribute this potentiation of sparks to enhanced buffering of luminal [Ca\(^{2+}\)] by the chelators entrapped in the SR.

The effects of intraluminal buffers on spatiotemporal properties of sparks are summarized in Figure 4 (see also online Table 3). ADA (5 mmol/L) and citrate (20 mmol/L) were loaded into the SR by a 30-minute incubation; sparks were then measured within 3 minutes after washout of the chelators from the experimental chamber, as shown in Figure
The images of sparks in Figure 4A were obtained by averaging the brightest events (5% of all events in each group, with most likely localization in the center of the line-scan) measured in control as well as in the presence of luminal ADA and citrate. Similar to the effects on caffeine-induced Ca\(^{2+}\)/H\(_{11001}\) transients, the effects on Ca\(^{2+}\)/H\(_{11001}\) sparks were most pronounced with ADA, which has the highest capacity to buffer luminal Ca\(^{2+}\). The effects of the exogenous luminal buffers on the magnitude and time course of the sparks are further illustrated in Figure 4B. In the inset, the signals were scaled to the same peak amplitude to better illustrate the effects on rise times. In addition to increasing the amplitude and the overall duration of the signal, the luminal buffers dramatically increased the rise time of sparks (about 1.74- and 2.35-fold for citrate and ADA, respectively). Figure 4D shows the effects of luminal buffers on the distribution of spark rise times for all the events. Luminal citrate and ADA produced significant shifts in the distribution of spark rise times to the right. The duration of the rising phase of the spark has been shown to provide a good estimate of the duration of underlying Ca\(^{2+}\)/H\(_{11001}\) release. Therefore, the observed increase in duration of the rising phase of the events is a clear indication that the release was prolonged in the presence of the luminal buffers. The changes in Ca\(^{2+}\) release fluxes are further emphasized by the prolonged duration of the first derivatives of the fluorescent signals in the presence of the exogenous chelators in the SR (Figure 4C). The first derivative of the fluorescent signal can be considered to be an approximation of the rate of local Ca\(^{2+}\) release underlying the spark.\(^{20}\)

**Simulated Ca\(^{2+}\) Release**

To further evaluate the effects of luminal Ca\(^{2+}\) chelators on Ca\(^{2+}\) release, we used a theoretical model of Ca\(^{2+}\) release that simulates changes in local [Ca\(^{2+}\)] in both the cytosolic and luminal compartments. Because this approach relies on assumptions regarding binding and diffusion parameters for different Ca\(^{2+}\) binding molecules (online Table 1), it is intended only as a qualitative analysis tool. As shown in Figure 5, activation of the release unit for a fixed period of time (5 ms) produces a luminal to cytosolic Ca\(^{2+}\) flux and results in an increase in the local cytosolic [Ca\(^{2+}\)] and a decline in the local SR luminal [Ca\(^{2+}\)]. Increasing luminal Ca\(^{2+}\) buffering has only minor effects on the magnitude and time course of the local cytosolic Ca\(^{2+}\) transients and no effect on their rise time; however, the extent of depletion of local [Ca\(^{2+}\)]\(_{SR}\) is reduced substantially (Figures 5C and 5D, left traces). To approximately reproduce the experimentally observed increases in the magnitude and the time-to-peak of Ca\(^{2+}\) sparks in the presence of ADA and citrate, the release unit open time had to be increased about 3- and 4-fold, respectively (Figures 5C and 5D, right traces). Thus, our experimental and modeling results suggest that increased buffering profoundly influences the duration of local Ca\(^{2+}\) release fluxes, apparently by influencing RyR open probability. Another important point suggested by our simulations is...
that in addition to affecting release flux duration the exogenous buffers should also slow dramatically the recovery of luminal \([\text{Ca}^{2+}]_{\text{SR}}\) after release. Intuitively, this is expected because of the increased filling capacity of the SR. This would imply that if termination of local \([\text{Ca}^{2+}]_{\text{SR}}\) release is indeed controlled by luminal \([\text{Ca}^{2+}]_{\text{SR}}\), the presence of exogenous buffers in the SR should influence the apparent refractoriness of release sites by affecting the dynamics of recovery of local \([\text{Ca}^{2+}]_{\text{SR}}\) sparks.

**Repetitive Activity Induced by IpTxA**

To explore the effect of luminal buffers on the ability of release sites to fire repeatedly, we performed experiments with the high-affinity RyR activator Imperatoxin A (IpTxA). In bilayer experiments, IpTxA has been shown to activate skeletal and cardiac RyRs by inducing long-lived openings with 1/3 conductance, and by increasing the frequency of transitions from closed to fully open states\(^{21}\) (H.H. Valdivia and S. Györke, unpublished observations, 2002). In accordance with the single channel effects, the toxin induces \([\text{Ca}^{2+}]_{\text{SR}}\) sparks with long-lasting tails and generates repetitive sparks at individual release sites in skeletal muscle.\(^{22}\) We found that IpTxA has qualitatively similar effects on \([\text{Ca}^{2+}]_{\text{SR}}\) sparks in cardiac myocytes. Thus, IpTxA increased the overall frequency of events \(\sim\)3.5-fold, mainly by increasing the number of repetitive events at the same individual sites (Figure 6C); 14 of a total of 2381 events in the presence of IpTxA (10 nmol/L) for reference conditions as well as for luminal maleate or citrate. Maleate and citrate were loaded into the SR by \(\sim\)30 minutes incubation and sparks measured within 5 minutes after washout of the chelators from the cytosolic compartment.

An example of repetitive activity of a release site in the presence of 10 nmol/L IpTxA is shown in Figure 6A. Repeated events are apparently ignited by a single toxin-modified RyR that acts as a pacemaker for the whole release unit. Interestingly, the amplitude of the recurrent events exhibited high variability, presumably due to a variable number of channels involved in the events and variations in the local \([\text{Ca}^{2+}]_{\text{SR}}\). Loading the SR with 20 nmol/L maleate or citrate led to a dramatic prolongation of the time period between repetitive sparks induced by IpTxA (average interspark intervals increased 2.3- and 5-fold, respectively; Figure 6B). Accordingly, the overall frequency of events
was decreased by 38% and 54%, respectively (Figure 6C). At the same time, luminal chelators had only a small effect on the frequency of Ca\(^{2+}\) sparks measured in the absence of the toxin (Figure 6C). We attribute the differential effects of the luminal Ca\(^{2+}\) buffers on repeated IpTxA-induced sparks and spontaneous sparks to differences in the dynamics of local luminal [Ca\(^{2+}\)]. The capacity of IpTxA to activate repeatedly the same release sites depends on the rate of refilling of the Ca\(^{2+}\) storage sites, which is in turn influenced by binding of Ca\(^{2+}\) to luminal buffers. On the other hand, in the absence of IpTxA the frequency of sparks arising spontaneously at the same site is very low (P≈0.025 s\(^{-1}\) 100 μm\(^{-1}\), assuming a spark frequency of 5 s\(^{-1}\) 100 μm\(^{-1}\) in the whole line scan and ≈200 release sites contained in the volume scanned by the confocal microscope\(^7\)), and the Ca\(^{2+}\) storage sites have sufficient time to fully recharge even when the loading capacity of the stores is increased by the exogenous Ca\(^{2+}\) chelators. These results indicate that [Ca\(^{2+}\)]\(_{\text{SR}}\) strongly influences not only the termination of local release but also the restitution behavior of release sites after Ca\(^{2+}\) sparks in cardiac myocytes.

**Discussion**

How CICR is terminated is an outstanding question in the field of cardiac EC coupling. During the Ca\(^{2+}\) release process, [Ca\(^{2+}\)] on the cytosolic side of the SR increases, whereas [Ca\(^{2+}\)] in the SR lumen decreases. Accordingly, two types of mechanisms have been discussed in the literature to explain Ca\(^{2+}\) release termination: (1) binding of Ca\(^{2+}\) to inhibition sites on the RyR reduces channel activity through processes referred to as Ca\(^{2+}\)-dependent inactivation or adaptation\(^4\),\(^10\),\(^11\),\(^23\),\(^24\); and (2) dissociation of Ca\(^{2+}\) from luminal activation sites decreases RyR open probability by a process that can be termed luminal Ca\(^{2+}\)-dependent deactivation\(^25\),\(^26\).

In this study, we have loaded low-affinity Ca\(^{2+}\) buffers into the SR luminal compartment to probe the mechanisms responsible for termination of local Ca\(^{2+}\) release events, ie, Ca\(^{2+}\) sparks, in permeabilized rat ventricular myocytes. Such buffers should stabilize or “clamp” [Ca\(^{2+}\)]\(_{\text{SR}}\), thus enabling us to address the importance of changes in luminal versus cytosolic Ca\(^{2+}\) in termination of Ca\(^{2+}\) sparks. We found that buffering luminal [Ca\(^{2+}\)] dramatically increases the amplitude and rise time of sparks as well as the duration of local Ca\(^{2+}\) release fluxes underlying sparks, thus implying that duration of Ca\(^{2+}\) release depends on changes in [Ca\(^{2+}\)]\(_{\text{SR}}\). The buffers also reduced the frequency of repetitive activity induced at individual release sites by the high-affinity RyR activating peptide IpTxA, although they did not considerably affect the frequency of events occurring randomly throughout the cells. These results indicate that the restitution behavior, ie, “refractoriness,” of release sites after discharge is related to local depletion and refilling of SR Ca\(^{2+}\) storage sites by Ca\(^{2+}\). In whole-cell patch-clamped myocytes, the SR lost its ability to respond to I\(_{\text{Ca}}\) in a graded manner in the presence of SR-permeable Ca\(^{2+}\) buffers. The buffers also slowed the recovery of cell-averaged Ca\(^{2+}\) transients in experiments with two pulses. Taken together our results suggest that termination of Ca\(^{2+}\) release and the subsequent restitution behavior of release sites in cardiac muscle is controlled by local intra-SR [Ca\(^{2+}\)] regulating RyR openings.

Previous studies from our and other laboratories have shown that luminal Ca\(^{2+}\) enhances RyR activity by acting at sites localized at the luminal side of the RyR complex.\(^25\),\(^28\),\(^29\) The effects of luminal Ca\(^{2+}\) have been also observed in intact and permeabilized myocytes as increases in frequency of Ca\(^{2+}\) sparks on elevating the SR Ca\(^{2+}\) load.\(^3\),\(^16\),\(^30\) The luminal Ca\(^{2+}\) sensor have been shown to continuously regulate the functional activity of the SR Ca\(^{2+}\) stores by linking SR Ca\(^{2+}\) content to the activity of the RyRs.\(^16\),\(^29\) This control mechanism, although possessing a certain time lag (due to the dynamics of changing SR load), stabilizes Ca\(^{2+}\) cycling when either Ca\(^{2+}\) release or uptake is altered. For example, when the RyRs are inhibited by tetracaine, reduced leak leads to elevation of SR Ca\(^{2+}\) content.\(^16\),\(^31\) Increased luminal [Ca\(^{2+}\)]\(_{\text{SR}}\) then enhances RyR P\(_{\text{on}}\), countering the inhibitory action of tetracaine. In the present study, we have shown that a luminal Ca\(^{2+}\) sensor also regulates RyR activity in a more immediate fashion, directly accounting for or contributing to termination of CICR at both local and global levels.

Our finding that Ca\(^{2+}\) spark termination is governed by luminal Ca\(^{2+}\) reconciles a body of apparently contradicting results. Several studies have found that SR Ca\(^{2+}\) release is turned off during sustained Ca\(^{2+}\) trigger stimuli before the SR would exhaust its supply of Ca\(^{2+}\) (as assessed by caffeine).\(^11\),\(^23\) Consequently, it has been proposed that release termination is caused by mechanisms such as Ca\(^{2+}\)-dependent inactivation or adaptation of RyRs, which depend on elevation of cytosolic Ca\(^{2+}\), or channel activation per se, rather than by a loss of Ca\(^{2+}\) from the SR.\(^1\),\(^11\),\(^24\) The present study shows that even a partial Ca\(^{2+}\) depletion can terminate Ca\(^{2+}\) sparks, and that the apparent refractoriness after release can be ascribed to the time required for partially depleted SR to be refilled with Ca\(^{2+}\). It is important to note that the Ca\(^{2+}\) release channels on partial depletion of stores do not become absolutely refractory, ie, desensitized to the cytosolic Ca\(^{2+}\) trigger. Instead, changes in luminal [Ca\(^{2+}\)] reduce the sensitivity of the release channels to cytosolic [Ca\(^{2+}\)].\(^16\),\(^25\),\(^32\) leaving the channels potentially responsive to larger Ca\(^{2+}\) concentrations.

Therefore, our finding could account for the ability of Ca\(^{2+}\) stores to respond transiently to multiple incremental increases in the trigger signal, a phenomenon termed “quantal” or “adaptive” Ca\(^{2+}\) release. The mechanisms underlying this phenomenon, demonstrated in both ryanodine- and Ip\(_{\text{p}}\)-sensitive stores, have not been defined thus far, although several schemes have been discussed (eg, Fill et al\(^24\) and Koizumi et al\(^32\)), including Ca\(^{2+}\) release from functionally heterogeneous Ca\(^{2+}\) stores, inactivation/adaptation of RyRs, and control of RyR openings by Ca\(^{2+}\) within the lumen of the SR. Local [Ca\(^{2+}\)]\(_{\text{SR}}\) changes and the subsequent changes in RyR open probability could also account for our previous observations that the decay of back-calculated Ca\(^{2+}\) release fluxes underlying sparks increase with increasing SR Ca\(^{2+}\) load.\(^10\) Indeed, faster releases from more fully loaded SR Ca\(^{2+}\) stores could lead to a more rapid and synchronous activation of RyRs composing the release unit, resulting in a faster decline of luminal [Ca\(^{2+}\)]. Effects of luminal Ca\(^{2+}\) on RyR functional activity is also the most likely explanation for the differ-
ences in restitution behavior of the SR Ca\(^{2+}\) stores after their global versus local activation by photolysis of caged Ca\(^{2+}\), as suggested by DelPrincipe et al.\(^{26}\)

Wang et al\(^{13}\) demonstrated that 10- to 20-fold overexpression of calsequestrin leads to impaired Ca\(^{2+}\) release and a reduction of spontaneous Ca\(^{2+}\) sparks in transgenic mouse cardiomyocytes. The reasons for the discrepancies between our results and those with overexpressed calsequestrin are not known. We can speculate that in calsequestrin-overexpressing myocytes, the free [Ca\(^{2+}\)\(_{\text{ls}}\)] may be chronically lowered due to exceptionally heavy Ca\(^{2+}\) buffering, leading to depressed RyR activity. Considering a concentration of intra-SR Ca\(^{2+}\) sites of \(\approx 10\) mmol/L that is almost entirely due to the presence calsequestrin,\(^{2,12}\) a \(\approx 10\)- to 20-fold overexpression of this protein would result in an increase of concentration of intra-SR Ca\(^{2+}\) sites to \(\approx 100\) to 200 mmol/L. With such a massive Ca\(^{2+}\) buffering, it might be difficult to attain the steady-state free [Ca\(^{2+}\)\(_{\text{ls}}\)] characteristic of normal myocytes. However, it is also possible that the alterations of release in transgenic myocytes are caused by direct effects of calsequestrin on RyR\(^{13}\) and/or some nonspecific changes induced by protein overexpression.

In conclusion, in the present study, we have shown that termination of SR Ca\(^{2+}\) release and the subsequent restitution behavior of the SR Ca\(^{2+}\) release sites in cardiac muscle is governed by local intra-SR [Ca\(^{2+}\)] regulating RyR openings. Thus, our study may provide a solution to the longstanding problem in the field of cardiac EC coupling: how is CICR terminated? It is also relevant to understanding intracellular Ca\(^{2+}\) signaling in other cell types, including neurons, skeletal muscle fibers, and smooth muscle cells, where Ca\(^{2+}\) sparks and CICR have been described.

**Acknowledgments**

This work was supported by grants from the American Heart Association and the National Institutes of Health (HL 52620 and HL 03739).

**References**


Luminal Ca$^{2+}$ Controls Termination and Refractory Behavior of Ca$^{2+}$-Induced Ca$^{2+}$ Release in Cardiac Myocytes
Dmitry Terentyev, Serge Viatchenko-Karpinski, Héctor H. Valdivia, Ariel L. Escobar and Sandor Györke

Circ Res. 2002;91:414-420; originally published online August 8, 2002;
doi: 10.1161/01.RES.0000032490.04207.BD

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/91/5/414

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2002/08/28/91.5.414.DC1
ONLINE DATA SUPPLEMENT

Mathematical Model of Ca\textsuperscript{2+} Release

The local changes in cytosolic and luminal [Ca\textsuperscript{2+}] were simulated using a one-dimensional diffusion-reaction model of Ca\textsuperscript{2+} dynamics in the cytosolic and SR luminal spaces, derived from our previous models of Ca\textsuperscript{2+} handling.\textsuperscript{1,2} The geometry of the model consisted of two adjacent sections, representing the cytosolic and SR luminal spaces of a sarcomere, each sliced into N diffusionally connected sub-compartments. The exchange of Ca between the cytosolic and luminal sections occurred only via RyRs (located in the SR membrane between the first cytosolic and luminal compartments) and SR Ca\textsuperscript{2+} pumps (placed between cytosolic and luminal compartments in a central region of the simulation domain). SR Ca efflux was governed by the Ca\textsuperscript{2+} gradient across the SR membrane and SR Ca\textsuperscript{2+} release channel function (i.e. composite RyR conductance and duration). Ca\textsuperscript{2+} sparks were simulated as the result of Ca\textsuperscript{2+} release into a medium with fixed and diffusible Ca\textsuperscript{2+} binding molecules, mimicking the cytoplasm. Changes in local luminal Ca\textsuperscript{2+} were simulated as the result of Ca\textsuperscript{2+} efflux from a medium with fixed endogenous Ca\textsuperscript{2+} buffers (calsequestrin) and diffusible exogenous buffers (citrate, maleate or ADA) mimicking the SR in our experiments. All dynamic equations, parameter values and initial conditions for our model are presented below.

Ca\textsuperscript{2+} distribution in the myoplasm

To describe the changes in local Ca\textsuperscript{2+} concentration, the cytosolic space was divided into four regions: 1) dyadic compartment containing the sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release channels/ryanodine receptors (RyRs) as well as diffusible Ca\textsuperscript{2+} buffers and the Ca\textsuperscript{2+} indicator dye; 2) intermediate region composed of l compartments containing only diffusible Ca\textsuperscript{2+} buffers and the dye; 3) central region composed of m compartments; in addition to
diffusible buffers and the dye, this region contained also fixed buffers (troponin) and the SR Ca\(^{2+}\) pump was located here; and 4) distant region composed of \(n\) compartments, which similar to the intermediate region, contained only diffusible buffers and the dye.

a) [Ca\(^{2+}\)] changes in the dyadic space (compartment 0):

The absolute changes in [Ca\(^{2+}\)] in this compartment were given by the difference between the influx and efflux of Ca\(^{2+}\) as follows:

\[
\frac{\partial [\text{Ca}^{2+}] (t,0)}{\partial t} = J_{\text{in}}^{Ca} (t,0) - J_{\text{out}}^{Ca} (t,0)
\]  

where \(J_{\text{in}}^{Ca} (t,0)\) is the net Ca\(^{2+}\) influx and \(J_{\text{out}}^{Ca} (t,0)\) is the net Ca\(^{2+}\) efflux from compartment 0 at time \(t\). The net Ca\(^{2+}\) influx was given by:

\[
J_{\text{in}}^{Ca} (t,0) = J_{\text{in}}^{Ca} (t,0)_{\text{RyR}} + J_{\text{in}}^{Ca} (t,0)_{B} + J_{\text{in}}^{Ca} (t,0)_{D} + J_{\text{in}}^{Ca} (t,0)_{\text{diff}}
\]  

where \(J_{\text{in}}^{Ca} (t,0)_{\text{RyR}}\) is the flux due to Ca\(^{2+}\) release via the RyRs, \(J_{\text{in}}^{Ca} (t,0)_{B}\) is the flux due to Ca\(^{2+}\) released from mobile intracellular buffers (a general term that combines the properties of several Ca\(^{2+}\)-binding molecules such as ATP and calmodulin), \(J_{\text{in}}^{Ca} (t,0)_{D}\) is the flux due to Ca\(^{2+}\) unbinding from the dye, and \(J_{\text{in}}^{Ca} (t,0)_{\text{diff}}\) is the flux due to diffusion of Ca\(^{2+}\) from the first compartment of the intermediate region into the dyadic compartment. These components were defined as follows:

\[
J_{\text{in}}^{Ca} (t,0)_{\text{RyR}} = P_{\text{RyR}} (t) \cdot \langle [\text{Ca}^{2+}]_{SR} (t,0) - [\text{Ca}^{2+}] (t,0) \rangle
\]  

\[
J_{\text{in}}^{Ca} (t,0)_{B} = k_{\text{affB}} \cdot \text{CaB}(t,0)
\]  

\[
J_{\text{in}}^{Ca} (t,0)_{D} = k_{\text{affD}} \cdot \text{CaD}(t,0)
\]  

\[
J_{\text{in}}^{Ca} (t,0)_{\text{diff}} = \frac{D_{\text{Ca}}}{dx^{2}}[\text{Ca}^{2+}] (t,1)
\]
Here, $P_{\text{RyR}}(t)$ is the Ca$^{2+}$ permeability of the RyR channel, $[\text{Ca}^{2+}]_{(t, SR0)}$ is the local free Ca$^{2+}$ concentration in the SR lumen at the inner mouth of the RyR, and $[\text{Ca}^{2+}]_{(t,0)}$ is the cytosolic [Ca$^{2+}$] in compartment 0 at time $t$. The term $P_{\text{RyR}}(t)$ was defined as:

$$P_{\text{RyR}}(t) = P_{\text{RyR,max}} \cdot \text{Pulse}(\text{delay, dur, } t)$$  \hspace{1cm} (7)

where $P_{\text{RyR,max}}$ is the maximum RyR Ca$^{2+}$ per unit of time, delay is the delay before the opening of the conductance, dur is the duration of conductance opening, and Pulse is a function defined as:

$$\begin{cases} 
\text{Pulse}(\text{delay, dur, } t) = 0 & \iff t < \text{delay} \\
\text{Pulse}(\text{delay, dur, } t) = 1 & \iff t \geq \text{delay} \\
\text{Pulse}(\text{delay, dur, } t) = 0 & \iff t \geq \text{delay} + \text{dur}
\end{cases}$$ \hspace{1cm} (8)

Further, $\text{CaB}(t,0)$ and $\text{CaD}(t,0)$ are the concentrations of Ca$^{2+}$ bound to mobile buffers and to the Ca$^{2+}$ indicator in compartment 0, $k_{\text{offB}}$ and $k_{\text{offD}}$ are the rate constants of dissociation of Ca$^{2+}$ from the mobile buffers and the indicator, respectively, $D_{\text{Ca}}$ is the Ca$^{2+}$ diffusion coefficient, $dx$ the thickness of compartment 0, and $[\text{Ca}^{2+}]_{(t,1)}$ is the Ca$^{2+}$ concentration in the first non-dyadic compartment.

The net Ca$^{2+}$ efflux from compartment 0, $J_{\text{out}}(t,0)$ was given by three components, i.e. unbinding from mobile buffers and the indicator, and Ca$^{2+}$ movement by diffusion:

$$J_{\text{out}}(t,0) = J_{\text{out}}^{\text{Ca}}(t,0)_B + J_{\text{out}}^{\text{Ca}}(t,0)_D + J_{\text{out}}^{\text{Ca}}(t,0)_{\text{Diff}}$$ \hspace{1cm} (9)

The Ca$^{2+}$ fluxes due to Ca$^{2+}$ binding to mobile buffers and to the indicator were defined as:

$$J_{\text{out}}^{\text{Ca}}(t,0)_B = k_{\text{onB}} \cdot [\text{Ca}^{2+}]_{(t,0)} \cdot [\text{B}_{\text{in}}(t,0) - \text{CaB}(t,0)]$$ \hspace{1cm} (10)

and

$$J_{\text{out}}^{\text{Ca}}(t,0)_D = k_{\text{onD}} \cdot [\text{Ca}^{2+}]_{(t,0)} \cdot [\text{D}_{\text{in}}(t,0) - \text{CaD}(t,0)]$$ \hspace{1cm} (11)
Here, $B_{tot}(t,0)$ and $D_{tot}(t,0)$ are the total concentrations of the mobile Ca$^{2+}$ buffer and indicator, respectively, in compartment 0; $k_{onB}$ and $k_{onD}$ are the association rate constants for the mobile buffer and the indicator, respectively. The Ca$^{2+}$ efflux due to diffusion was given by:

$$J^{Ca}_{out}(t,0)_{Diff} = \frac{D_{Ca}}{d^2} \bullet \left[ Ca^{2+} \right](t,0) \tag{12}$$

The diffusion of the mobile buffers and the Ca$^{2+}$ indicator in and out of the dyadic space was implemented as follows:

$$\frac{\partial \left[ CaB \right](t,0)}{\partial t} = J^{CaB}_{in}(t,0) - J^{CaB}_{out}(t,0) \tag{13}$$

and

$$\frac{\partial \left[ CaD \right](t,0)}{\partial t} = J^{CaD}_{in}(t,0) - J^{CaD}_{out}(t,0) \tag{14}$$

where

$$J^{CaB}_{in}(t,0) = J^{CaB}_{in}(t,0)_B + J^{CaB}_{in}(t,0)_{Diff} \tag{15}$$

$$J^{CaB}_{out}(t,0) = J^{CaB}_{out}(t,0)_B + J^{CaB}_{out}(t,0)_{Diff} \tag{16}$$

and

$$J^{CaD}_{in}(t,0) = J^{CaD}_{in}(t,0)_D + J^{CaD}_{in}(t,0)_{Diff} \tag{17}$$

$$J^{CaD}_{out}(t,0) = J^{CaD}_{out}(t,0)_D + J^{CaD}_{out}(t,0)_{Diff} \tag{18}$$

Specifically,

$$J^{CaB}_{in}(t,0)_B = k_{onB} \bullet \left[ B_{tot}(t,0) - CaB(t,0) \right] = J^{Ca}(t,0)_B \tag{19}$$

$$J^{CaD}_{in}(t,0)_D = k_{onD} \bullet \left[ D_{tot}(t,0) - CaD(t,0) \right] = J^{Ca}(t,0)_D \tag{20}$$

$$J^{CaB}_{in}(t,0)_{Diff} = \frac{D_{CaB}}{dx^2} \left[ CaB \right](t,1) \tag{21}$$

$$J^{CaD}_{in}(t,0)_{Diff} = \frac{D_{CaD}}{dx^2} \left[ CaD \right](t,1) \tag{22}$$

and
\[ J_{\text{out}}^{\text{CaB}}(t,0)_B = k_{\text{diffB}} \cdot \text{CaB}(t,0) = J_{\text{in}}^{\text{Ca}}(t,0)_B \quad (23) \]

\[ J_{\text{out}}^{\text{CaD}}(t,0)_D = k_{\text{diffD}} \cdot \text{CaD}(t,0) = J_{\text{in}}^{\text{Ca}}(t,0)_D \quad (24) \]

\[ J_{\text{out}}^{\text{CaB}}(t,0)_{\text{Diff}} = \frac{D_{\text{CaB}}}{dx^2} \cdot [\text{CaB}](t,0) \quad (25) \]

\[ J_{\text{out}}^{\text{CaD}}(t,0)_{\text{Diff}} = \frac{D_{\text{CaD}}}{dx^2} \cdot [\text{CaD}](t,0) \quad (26) \]

The quantities \( D_{\text{CaB}} \) and \( D_{\text{CaD}} \) are the diffusion coefficients for the \( \text{Ca}^{2+} \)-bound buffer and \( \text{Ca}^{2+} \)-bound indicator, respectively. Assuming that the \( \text{Ca}^{2+} \)-free and bound forms of the buffer and indicator molecules diffuse with the same speed, the total concentration of the mobile buffer and the indicator will be constant as a function of time in this compartment:

\[ B_{\text{tot}}(t,0) = B_{\text{free}}(t,0) - \text{CaB}(t,0) = B_{\text{tot-initial}}(0) = \text{constant} \quad (27) \]

\[ D_{\text{tot}}(t,0) = D_{\text{free}}(t,0) - \text{CaD}(t,0) = D_{\text{tot-initial}}(0) = \text{constant} \quad (28) \]

b) \([\text{Ca}^{2+}] \) changes in the intermediate compartments (I to I+I):

The change in \( \text{Ca}^{2+} \) concentration in these compartments was computed as the difference between \( \text{Ca}^{2+} \) influx and \( \text{Ca}^{2+} \) efflux as follows:

\[ \frac{\partial[\text{Ca}^{2+}](t,j)}{\partial t} \bigg|_{j=1}^{j=I+1} = J_{\text{in}}^{\text{Ca}}(t,j) - J_{\text{out}}^{\text{Ca}}(t,j) \quad (29) \]

The \( \text{Ca}^{2+} \) influx to these compartments was given by:

\[ J_{\text{in}}^{\text{Ca}}(t,j) = J_{\text{in}}^{\text{Ca}}(t,j)_B + J_{\text{in}}^{\text{Ca}}(t,j)_D + J_{\text{in}}^{\text{Ca}}(t,j)_{\text{Diff}} \quad (30) \]

Particularly,

\[ J_{\text{in}}^{\text{Ca}}(t,j)_B = k_{\text{diffB}} \cdot \text{CaB}(t,j) \quad (31) \]

\[ J_{\text{in}}^{\text{Ca}}(t,j)_D = k_{\text{diffD}} \cdot \text{CaD}(t,j) \quad (32) \]

\[ J_{\text{in}}^{\text{Ca}}(t,j)_{\text{Diff}} = \frac{D_{\text{Ca}}}{dx^2} \cdot [\text{Ca}^{2+}](t,j-1) + \frac{D_{\text{Ca}}}{dx^2} \cdot [\text{Ca}^{2+}](t,j+1) \quad (33) \]
where \([Ca^{2+}]_{(t, j-1)}\) and \([Ca^{2+}]_{(t, j+1)}\) are the Ca\(^{2+}\) concentrations in compartments contiguous to compartment \(j\). Efflux from each intermediate compartment was given by Ca\(^{2+}\) binding and diffusion:

\[
J^{Ca\_out}_{out}(t, j) = J^{Ca\_out}_{out}(t, j)_{B} + J^{Ca\_out}_{out}(t, j)_{D} + J^{Ca\_out}(t, j)_{Diff} \quad (34)
\]

Specifically,

\[
J^{Ca\_out}_{out}(t, j)_{B} = k_{onB} \cdot [Ca^{2+}]_{(t, j)} \cdot [B_{Tot\_j}(j) - CaB(t, j)] \quad (35)
\]

\[
J^{Ca\_out}_{out}(t, j)_{D} = k_{onD} \cdot [Ca^{2+}]_{(t, j)} \cdot [D_{Tot\_j}(j) - CaD(t, j)] \quad (36)
\]

and

\[
J^{Ca\_out}(t, j)_{Diff} = 2 \cdot \frac{D_{Ca\_out}}{dx^2} \cdot [Ca^{2+}]_{(t, j)} \quad (37)
\]

Similar to the case with the dyadic compartment, the indicator and the mobile buffers could react and diffuse:

\[
\frac{\partial [CaB \_j(t, j)]}{\partial t} = \left\{ k_{onB} \cdot \left[ B_{Tot\_j}(t, j) - CaB(t, j) \right] - k_{offB} \cdot CaB(t, j) \right\} + \frac{D_{CaB}}{dx^2} \left\{ CaB(t, j-1) - 2 \cdot CaB(t, j) + CaB(t, j+1) \right\} \quad (38)
\]

and

\[
\frac{\partial [CaD \_j(t, 0)]}{\partial t} = \left\{ k_{onD} \cdot \left[ D_{Tot\_j}(t, j) - CaD(t, j) \right] - k_{offD} \cdot CaD(t, j) \right\} + \frac{D_{CaD}}{dx^2} \left\{ CaD(t, j-1) - 2 \cdot CaD(t, j) + CaD(t, j+1) \right\} \quad (39)
\]

with

\[
B_{Tot\_j}(t, j) = B_{Tot\_initial\_j} = \text{constant} \quad (40)
\]

\[
D_{Tot\_j}(t, j) = D_{Tot\_initial\_j} = \text{constant} \quad (41)
\]

c) [Ca\(^{2+}\)] changes in the central compartments (compartment \(l+1\) to \(l+n+1\)):

The change in Ca\(^{2+}\) concentration in these compartments was given by:
\[
\frac{\partial [Ca^{2+}](t, j)}{\partial t} \bigg|_{t+1} = J_{in}^{Ca}(t, j) - J_{out}^{Ca}(t, j)
\]  

(42)

Ca\(^{2+}\) influx into these compartments was consisted of four terms:

\[
J_{in}^{Ca}(t, j) = J_{in}^{Ca}(t, j)_B + J_{in}^{Ca}(t, j)_D + J_{in}^{Ca}(t, j)_T + J_{in}^{Ca}(t, j)_{Diff}
\]  

(43)

The expressions for calculating \(J_{in}^{Ca}(t, j)_B\), \(J_{in}^{Ca}(t, j)_D\) and \(J_{in}^{Ca}(t, j)_{Diff}\) were identical to those described in section b. The new term \(J_{in}^{Ca}(t, j)_T\) represents unbinding of Ca\(^{2+}\) from troponin that is present in these compartments as an immobile buffer. It was defined as:

\[
J_{in}^{Ca}(t, j)_T = k_{eff} \cdot CaT(t, j)
\]

(44)

The Ca\(^{2+}\) efflux term comprised five individual components due to Ca\(^{2+}\) binding to mobile and immobile buffers and to the dye, Ca\(^{2+}\) diffusion to neighboring compartments, and Ca\(^{2+}\) extrusion by the SR Ca\(^{2+}\) pump.

\[
J_{out}^{Ca}(t, j) = J_{out}^{Ca}(t, j)_B + J_{out}^{Ca}(t, j)_D + J_{out}^{Ca}(t, j)_T + J_{out}^{Ca}(t, j)_{Diff} + J_{out}^{Ca}(t, j)_{Pump}
\]  

(45)

In particular,

\[
J_{out}^{Ca}(t, j)_{Pump} = Rate_{Pump} \cdot \left[ \frac{CaPump_{myo}(t, j)}{Pump_{Tot}(j)} \right] \cdot \left[ 1 - \frac{CaPump_{Lum}(t, j)}{Pump_{Tot}(j)} \right] \cdot [Ca^{2+}](t, j)
\]  

(46)

In this expression, \(J_{out}^{Ca}(t, j)_{Pump}\) is the magnitude of the Ca\(^{2+}\) efflux produced by the sarcoplasmic reticulum Ca\(^{2+}\) pump. This flux was limited by the maximal transfer rate, \(Rate_{Pump}\). The flux could be activated by the fraction of occupied myoplasmic Ca\(^{2+}\) binding sites, \(\frac{CaPump_{myo}(t, j)}{Pump_{Tot}(j)}\)

and inhibited by the fraction of occupied luminal Ca\(^{2+}\) binding sites, \(\frac{CaPump_{Lum}(t, j)}{Pump_{Tot}(j)}\). The
occupation of the myoplasmic Ca\(^{2+}\) binding sites was described by a second order kinetic scheme:

\[
\frac{d[Pump_{myo}(t,j)]}{dt} = -2 \cdot k_{onPump}^{myo} \cdot [Ca^{2+}](t,j) \cdot Pump_{myo}(t,j) + k_{offPump}^{myo} \cdot CaPump_{myo}(t,j) \tag{47}
\]

\[
\frac{d[CaPump_{myo}(t,j)]}{dt} = 2 \cdot k_{onPump}^{myo} \cdot [Ca^{2+}](t,j) \cdot Pump_{myo}(t,j) - \left( k_{offPump}^{myo} + k_{onPump}^{myo} \cdot [Ca^{2+}] \right) \cdot CaPump_{myo}(t,j) + 2 \cdot k_{offPump}^{myo} \cdot Ca_2Pump_{myo}(t,j) \tag{48}
\]

\[
\frac{d[Ca_2Pump_{myo}(t,j)]}{dt} = k_{onPump}^{myo} \cdot [Ca^{2+}](t,i) \cdot CaPump(t,i) - 2 \cdot k_{offPump}^{myo} \cdot Ca_2Pump(t,i) \tag{49}
\]

\[Pump_{Tot}(j) = Pump_{myo}(t,j) + CaPump_{myo}(t,j) + Ca_2Pump_{myo}(t,j) \tag{50}\]

Here, \(Pump_{myo}(t,j), CaPump_{myo}(t,j)\) and \(Ca_2Pump_{myo}(t,j)\) are different kinetic states in which no Ca, one Ca or two Ca ions are bound to the myoplasmic regulatory sites. The quantities \(k_{onPump}^{myo}\) and \(k_{offPump}^{myo}\) are the association and dissociation rate constants for Ca\(^{2+}\) binding to the cytosolic side of the pump. The inhibition of the pump at luminal sites was a first order process.

\[
\frac{d[Pump_{Lam}(t,j)]}{dt} = -k_{onPump}^{Lam} \cdot [Ca^{2+}]_{SR}(t,j) \cdot Pump_{Lam}(t,j) + k_{offPump}^{Lam} \cdot CaPump_{Lam}(t,j) \tag{51}
\]

\[
\frac{d[CaPump_{Lam}(t,j)]}{dt} = k_{onPump}^{Lam} \cdot [Ca^{2+}]_{SR}(t,j) \cdot Pump_{Lam}(t,j) - k_{offPump}^{Lam} \cdot CaPump_{Lam}(t,j) \tag{52}
\]

\[Pump_{Tot}(j) = Pump_{Lam}(t,j) + CaPump_{Lam}(t,j) \tag{53}\]

\(Pump_{Lam}(t,j)\) and \(CaPump_{Lam}(t,j)\) represent the Ca\(^{2+}\) free and Ca\(^{2+}\) bound states of the pump at the luminal regulatory site, \(k_{onPump}^{Lam}\) and \(k_{offPump}^{Lam}\) are the association and dissociation rate constants for the luminal site.

Finally, \([Ca^{2+}]\) in the central compartments could decrease through binding to troponin.
\[ J_{\text{out}}^{\text{Ca}}(t,j)_{S} = k_{\text{out}} \cdot [Ca^{2+}]_{\text{in}}(t,j) \cdot [T_{\text{Ca}}(j) - CaT(t,j)] \] (54)

As described for the intermediate compartments, the mobile buffers and the indicator were able to diffuse between the compartments in both Ca\(^{2+}\)-free and Ca\(^{2+}\)-bound forms.

d) \textbf{[Ca\(^{2+}\)] changes in the distant compartments (from } l+m+1 \text{ to } l+m+n+1):}

The dynamics of Ca\(^{2+}\), mobile buffers and the indicator were the same as described for the intermediate compartments (b). In the last compartment a reflection condition was set in order integrate the model and the model was assumed to be symmetric with respect to the position of the release sites.

\textbf{Ca\(^{2+}\) distribution in the sarcoplasmic reticulum.}

In this model, the sarcoplasmic reticulum consisted of \( l+m+n+1 \) diffusonally connected compartments. All these compartments contained an exogenous mobile Ca\(^{2+}\) buffer (\( M \)) and the fixed Ca\(^{2+}\) binding protein calsequestrin (\( Q \)). The general expression for calculating the free Ca\(^{2+}\) concentration in the luminal compartments was given by:

\[
\frac{\partial [Ca^{2+}]_{SR}(t,j)}{\partial t} \bigg|_{0}^{l+m+n+1} = J_{\text{in}}^{\text{Ca}}(t,j)_{SR} - J_{\text{out}}^{\text{Ca}}(t,j)_{SR} = \left\{ J_{\text{in}}^{\text{Ca}}(t,j)_{M} - J_{\text{out}}^{\text{Ca}}(t,j)_{M} \right\} + \left\{ J_{\text{in}}^{\text{Ca}}(t,j)_{Q} - J_{\text{out}}^{\text{Ca}}(t,j)_{Q} \right\} + \left\{ J_{\text{in}}^{\text{Ca}}(t,j)_{\text{diff}} - J_{\text{out}}^{\text{Ca}}(t,j)_{\text{diff}} \right\} + \left\{ J_{\text{in}}^{\text{Ca}}(t,j)_{\text{pump}} - J_{\text{out}}^{\text{Ca}}(t,j)_{\text{pump}} \right\} - J_{\text{out}}^{\text{Ca}}(t,0)_{SR} \] (55)
And

\[ J^{Ca}_{\text{out}}(t,0)_{\text{RyR}}^{SR} = P_{\text{RyR}}(t) \cdot \left\{ [Ca^{2+}]_{\text{SR}}(t,0) - [Ca^{2+}]_{\text{SR}}(t,0) \right\} \cdot \text{vol}_{\text{myo}/\text{SR}} \]  

(56)

\[ J^{Ca}_{\text{in}}(t,j)_{M} - J^{Ca}_{\text{out}}(t,j)_{M} = k_{\text{onM}} \cdot CaM(t,j) - k_{\text{offM}} \cdot [Ca^{2+}]_{\text{SR}}(t,j) \cdot [M_{\text{Tot}}(j) - CaM(t,j)] \]  

(57)

\[ J^{Ca}_{\text{in}}(t,j)_{Q} - J^{Ca}_{\text{out}}(t,j)_{Q} = k_{\text{onQ}} \cdot CaQ(t,j) - k_{\text{offQ}} \cdot [Ca^{2+}]_{\text{SR}}(t,j) \cdot [Q_{\text{Tot}}(j) - CaQ(t,j)] \]  

(58)

\[ J^{Ca}_{\text{in}}(t,j)_{\text{diff}} - J^{Ca}_{\text{out}}(t,j)_{\text{diff}} = \frac{D_{Ca}}{dx^2} \cdot \left\{ [Ca^{2+}]_{\text{SR}}(t,j-1) - 2 \cdot [Ca^{2+}]_{\text{SR}}(t,j) + [Ca^{2+}]_{\text{SR}}(t,j+1) \right\} \]  

(59)

\[ J^{Ca}_{\text{in}}(t,j)_{\text{Pump}}^{SR} = \text{Rate}_{\text{Pump}} \cdot \left\{ \frac{\text{CaPump}_{\text{myo}}(t,j)}{\text{Pump}_{\text{Tot}}(j)} \right\} \cdot \left\{ 1 - \frac{\text{CaPump}_{\text{Lum}}(t,j)}{\text{Pump}_{\text{Tot}}(j)} \right\} \cdot [Ca^{2+}](t,j) \cdot \text{vol}_{\text{myo}/\text{SR}} \]  

(60)

In these expressions, \( k_{\text{onM}}, k_{\text{onQ}}, k_{\text{offM}} \) and \( k_{\text{offQ}} \) are the association and dissociation rate constants for the exogenous buffer and calsequestrin. \( \text{vol}_{\text{myo}/\text{SR}} \) is the ratio between the myoplasmic volume and the sarcoplasmic reticulum volume. \( Ca^{2+} \) efflux through RyRs, \( J^{Ca}_{\text{out}}(t,0)_{\text{RyR}}^{SR} \), took place only in the first compartment (i.e. facing the dyadic compartment) and \( Ca^{2+} \) uptake by the \( Ca^{2+} \) pump, \( J^{Ca}_{\text{in}}(t,0)_{\text{Pump}}^{SR} \), occurred in the central compartments (from 1 to \( l+m \)).

The mobile exogenous buffer was able to diffuse according to

\[ \frac{\partial [CaM](t,j)}{\partial t} \bigg|_{t=0}^{l+m+a+1} = \left\{ k_{\text{onM}} \cdot [M_{\text{Tot}}(t,j) - CaM(t,j)] - k_{\text{offM}} \cdot CaM(t,j) \right\} + \]  

\[ \frac{D_{Ca}}{dx^2} \cdot \left\{ CaM(t,j-1) - 2 \cdot CaM(t,j) + CaM(t,j+1) \right\} \]  

and

\[ M_{\text{Tot}}(t,j) = M_{\text{Tot - initial}}(j) = \text{constant} \]  

(61)

All equations were numerical integrated using a finite difference approximation (Euler method). The initial values for the state variables were calculated using the inversion of the \( Q \)-matrix procedure.\(^1\) The boundary conditions were established to satisfy equations (1) and (55) for the first cytosolic and luminal compartments respectively and a Neumann’s boundary condition\(^7\)
was implemented for the last cytosolic and luminal compartments. The code was written in G language, LabView 6 (National Instruments, Austin, TX).

References:


Fig. 1. The effects of intracellular dialysis with low-affinity Ca chelators on recovery of EC coupling. A. Paired-pulse experiments, in which a pre-pulse is followed by a test-pulse at various time intervals, were carried out to delineate the time course of recovery of $I_{Ca}$ and intracellular Ca transients. The superimposed traces are records of $I_{Ca}$ and Ca transients acquired during two sequential 200 ms pulses to 0 mV, spaced at different time intervals, in a control myocyte and in myocytes dialyzed with maleate, citrate, or dn-BAPTA. The chelators were included into the pipette solutions at a concentration of 20 mmol/L (pCa 7, 0.1 mmol/L EGTA). The holding potential was $-50$ mV. B, Relative peak Ca transients and $I_{Ca}$ amplitudes (upper and lower panels, respectively) during the test-pulse plotted as a function of interpulse interval for control myocytes and for cells dialyzed with 20 mmol/L dn-BAPTA, maleate, or citrate (as indicated by the labels). Data are presented as means ± SE from 5-6 different experiments. Under baseline conditions (i.e. in the absence of chelators), the recovery of steady-state amplitudes of $I_{Ca}$ and Ca occurred with a similar rate (< 500 ms), suggesting that the restitution of Ca release might be limited by the recovery of $I_{Ca}$ from inactivation. Dialysis of the cells with maleate or citrate dramatically slowed the rate of restitution of Ca transients, without altering considerably the time-course of $I_{Ca}$ recovery. The presumably SR-impermeable Ca buffer dn-BAPTA had no significant impact on restitution of Ca release. This result may be an indication that the changes of release restitution in the presence of citrate and maleate were not due to the cytosolic effects of these buffers, but rather to their actions at the luminal side of the SR.
Table 1. Model parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[Ca^{2+}]_{ri}$</td>
<td>Resting myoplasmic $[Ca^{2+}]$</td>
<td>0.10</td>
<td>μM</td>
</tr>
<tr>
<td>$D_{Ca}$</td>
<td>$Ca^{2+}$ diffusion coefficient</td>
<td>0.25*</td>
<td>μM²/ms</td>
</tr>
<tr>
<td>$D_{Tot}$</td>
<td>Total indicator concentration</td>
<td>50</td>
<td>μM</td>
</tr>
<tr>
<td>$k_{emD}$</td>
<td>Indicator $Ca^{2+}$ association rate constant</td>
<td>0.08*</td>
<td>μM⁻¹·ms⁻¹</td>
</tr>
<tr>
<td>$k_{offD}$</td>
<td>Indicator $Ca^{2+}$ dissociation rate constant</td>
<td>0.09*</td>
<td>ms⁻¹</td>
</tr>
<tr>
<td>$D_{CaD}$</td>
<td>$Ca^{2+}$-indicator diffusion coefficient</td>
<td>0.02*</td>
<td>μm²/ms</td>
</tr>
<tr>
<td>$B_{Tot}$</td>
<td>Total mobile cytosolic buffer concentration</td>
<td>1000</td>
<td>μM</td>
</tr>
<tr>
<td>$k_{onB}$</td>
<td>Cytosolic buffer $Ca^{2+}$ association rate constant</td>
<td>0.01</td>
<td>μM⁻¹·ms⁻¹</td>
</tr>
<tr>
<td>$k_{offB}$</td>
<td>Cytosolic buffer $Ca^{2+}$ dissociation rate constant</td>
<td>0.01</td>
<td>ms⁻¹</td>
</tr>
<tr>
<td>$D_{CaB}$</td>
<td>$Ca^{2+}$-cytosolic buffer diffusion coefficient</td>
<td>0.02</td>
<td>μm²/ms</td>
</tr>
<tr>
<td>$T_{tot}$</td>
<td>Total troponin concentration</td>
<td>70*</td>
<td>μM</td>
</tr>
<tr>
<td>$k_{onT}$</td>
<td>Troponin $Ca^{2+}$ association rate constant</td>
<td>0.039*</td>
<td>μM⁻¹·ms⁻¹</td>
</tr>
<tr>
<td>$k_{offT}$</td>
<td>Troponin $Ca^{2+}$ dissociation rate constant</td>
<td>0.02*</td>
<td>ms⁻¹</td>
</tr>
<tr>
<td>$[Ca^{2+}]_{SR}$</td>
<td>Resting sarcoplasmic reticulum $[Ca^{2+}]$</td>
<td>1000+</td>
<td>μM</td>
</tr>
<tr>
<td>$Q_{Tot}$</td>
<td>Total calsequestrin concentration</td>
<td>10000+</td>
<td>μM</td>
</tr>
<tr>
<td>$k_{onQ}$</td>
<td>Calsequestrin $Ca^{2+}$ association rate constant</td>
<td>0.0001</td>
<td>μM⁻¹·ms⁻¹</td>
</tr>
<tr>
<td>$k_{offQ}$</td>
<td>Calsequestrin dissociation rate constant</td>
<td>0.1</td>
<td>ms⁻¹</td>
</tr>
<tr>
<td>$k_{onC}$</td>
<td>Citrate $Ca^{2+}$ association rate constant</td>
<td>0.001</td>
<td>μM⁻¹·ms⁻¹</td>
</tr>
<tr>
<td>$k_{offC}$</td>
<td>Citrate dissociation rate constant</td>
<td>0.14</td>
<td>ms⁻¹</td>
</tr>
<tr>
<td>$k_{onADA}$</td>
<td>ADA $Ca^{2+}$ association rate constant</td>
<td>0.02</td>
<td>μM⁻¹·ms⁻¹</td>
</tr>
<tr>
<td>$k_{offADA}$</td>
<td>ADA dissociation rate constant</td>
<td>11.2</td>
<td>ms⁻¹</td>
</tr>
<tr>
<td>$D_{X}$</td>
<td>Compartment length</td>
<td>0.1</td>
<td>μm</td>
</tr>
<tr>
<td>$N$</td>
<td>Total number of compartments</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>$L$</td>
<td>Number of compartments in intermediate region</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>$M$</td>
<td>Number of compartments in central region</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>$N$</td>
<td>Number of compartments in final region</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>$\delta t$</td>
<td>Integration time step</td>
<td>0.0008</td>
<td>ms</td>
</tr>
<tr>
<td>$Pump_{Tot}$</td>
<td>Concentration of pumps per compartment</td>
<td>1</td>
<td>μM</td>
</tr>
<tr>
<td>$Rate_{Pump}$</td>
<td>Pump maximal transfer rate</td>
<td>1</td>
<td>ms⁻¹</td>
</tr>
<tr>
<td>$k_{myo_{inPump}}$</td>
<td>Pump myoplasmic site $Ca^{2+}$ association rate constant</td>
<td>0.02</td>
<td>μM⁻¹·ms⁻¹</td>
</tr>
<tr>
<td>$k_{myo_{offPump}}$</td>
<td>Pump myoplasmic site $Ca^{2+}$ dissociation rate constant</td>
<td>0.04</td>
<td>ms⁻¹</td>
</tr>
<tr>
<td>$k_{Lum_{inPump}}$</td>
<td>Pump luminal site $Ca^{2+}$ association rate constant</td>
<td>0.02*</td>
<td>μM⁻¹·ms⁻¹</td>
</tr>
<tr>
<td>$k_{Lum_{offPump}}$</td>
<td>Pump luminal site $Ca^{2+}$ dissociation rate constant</td>
<td>20*</td>
<td>ms⁻¹</td>
</tr>
</tbody>
</table>

* ref. 4; ^ ref. 5; ^ ref. 6.
Table. 2. Effects of intracellular dialysis with low affinity Ca chelators on parameters of \( I_{Ca} \) and Ca transients in cardiac myocytes.

<table>
<thead>
<tr>
<th></th>
<th>( I_{Ca} )</th>
<th>( I_{Ca} )-induced Ca transients</th>
<th>Caffeine-induced Ca transients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amplitude (pA)</td>
<td>Decay-time (ms)</td>
<td>Amplitude (F/Fo)</td>
</tr>
<tr>
<td>Control</td>
<td>323 ± 93</td>
<td>24.2 ± 2.7</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>Maleate</td>
<td>343 ± 83</td>
<td>34.2 ± 4.5</td>
<td>3.6 ± 0.3*</td>
</tr>
<tr>
<td>Citrate</td>
<td>349 ± 110</td>
<td>65.4 ± 6.6**</td>
<td>4.5 ± 0.2**</td>
</tr>
<tr>
<td>Dn-Bapta</td>
<td>330 ± 85</td>
<td>45.8 ± 5.7*</td>
<td>2.3 ± 0.2</td>
</tr>
</tbody>
</table>

\( I_{Ca} \) and Ca transients were measured during depolarizing pulses to 0 mV from a holding potential of -50 mV in control myocytes and in myocytes dialyzed with 20 mmol/L maleate, citrate, or dN-BAPTA. Caffeine (20 mmol/L) was applied to assess the amount of releasable Ca. Data are presented as means ± SE from 16-20 different experiments. Rise- and decay-times of the current and fluorescence signals were measured at their half-maximal amplitude. *p< 0.02, or **p<0.0001 vs. values for control myocytes.
Table 3. Effects of SR luminal citrate and ADA on properties of spontaneous Ca sparks in permeabilized cardiomyocytes.

<table>
<thead>
<tr>
<th></th>
<th>Amplitude (F/F₀)</th>
<th>Rise-time (ms)</th>
<th>Duration (ms)</th>
<th>Width (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.60 ± 0.01</td>
<td>7.9 ± 0.2</td>
<td>16.8 ± 0.3</td>
<td>1.59 ± 0.03</td>
</tr>
<tr>
<td>Citrate</td>
<td>1.82 ± 0.02**</td>
<td>8.8 ± 0.2*</td>
<td>18.2 ± 0.2**</td>
<td>1.78 ± 0.02**</td>
</tr>
<tr>
<td>ADA</td>
<td>2.12 ± 0.03**</td>
<td>11.3 ± 0.3**</td>
<td>21.7 ± 0.3**</td>
<td>1.92 ± 0.02**</td>
</tr>
</tbody>
</table>

Maleate and citrate were loaded into the SR by ~30 min incubation and sparks measured within 5 min after washout of the chelators from the cytosolic compartment. Data are presented as means ± SE from 350-450 events from different experiments. ^Duration and width of Ca sparks were measured at half-maximal amplitude. *p<0.002, or **p<0.0001 vs. values for control myocytes.
Figure 1

A) Control vs. dn-BAPTA with Maleate and Citrate treatments.

B) Graph showing $F_{T}/F_{P}$ vs. Interpulse interval (s).

C) Graph showing $I_{CaT}/I_{CaP}$ vs. Interpulse interval (s).

Legend:
- Control
- dn-BAPTA
- Maleate
- Citrate