Dynamic Calcium Movement Inside Cardiac Sarcoplasmic Reticulum During Release

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Rationale: Intra-sarcoplasmic reticulum (SR) free [Ca] ([Ca]SR) provides the driving force for SR Ca release and is a key regulator of SR Ca release channel gating during normal SR Ca release or arrhythmogenic spontaneous Ca release events. However, little is known about [Ca]SR spatiotemporal dynamics.

Objective: To directly measure local [Ca]SR with subsarcomeric spatiotemporal resolution during both normal global SR Ca release and spontaneous Ca sparks and to evaluate the quantitative implications of spatial [Ca]SR gradients.

Methods and Results: Intact and permeabilized rabbit ventricular myocytes were subjected to direct simultaneous measurement of cytosolic [Ca] and [Ca]SR and FRAP (fluorescence recovery after photobleach). We found no detectable [Ca]SR gradients between SR release sites (junctional SR) and Ca uptake sites (free SR) during normal global Ca release, clear spatiotemporal [Ca]SR gradients during isolated Ca blinks, faster intra-SR diffusion in the longitudinal versus transverse direction, 3- to 4-fold slower diffusion of fluorophores in the SR than in cytosol, and that intra-SR Ca diffusion varies locally, dependent on local SR connectivity. A computational model clarified why spatiotemporal gradients are more detectable in isolated local releases versus global releases and provides a quantitative framework for understanding intra-SR Ca diffusion.

Conclusions: Intra-SR Ca diffusion is rapid, limiting spatial [Ca]SR gradients during excitation-contraction coupling. Spatiotemporal [Ca]SR gradients are apparent during Ca sparks, and these observations constrain models of dynamic Ca movement inside the SR. This has important implications for myocyte SR Ca handling, synchrony, and potentially arrhythmogenic spontaneous contraction. (Circ Res. 2011;108:847-856.)

Key Words: cardiac excitation-contraction coupling ■ sarcoplasmic reticulum ■ Ca sparks ■ Ca transport ■ ryanodine receptor

The sarcoplasmic reticulum (SR) is the main Ca storage organelle in cardiac myocytes. SR Ca release during excitation-contraction coupling (ECC) contributes the majority of Ca for cytosolic Ca transients and contractile activation.1,2 SR Ca load critically regulates SR Ca release during both ECC and spontaneous SR Ca release,3-5 which can cause delayed afterdepolarizations and arrhythmias. Local SR Ca depletion is believed to play a role in terminating SR Ca release6 and contribute to defective Ca handling in myocytes during heart failure and arrhythmias.

Local intra-SR Ca has important functional implications for control of physiological and pathophysiological SR Ca release. Slow intra-SR Ca diffusion would promote nonuniform distribution of SR Ca load and intra-SR free [Ca] ([Ca]SR) within the network. This could induce regions that are more likely to release Ca spontaneously or in response to a Ca trigger (in regions with higher [Ca]SR) versus regions that are less likely to release Ca (with lower local [Ca]SR). Fast intra-SR Ca diffusion, on the other hand, would tend to level intra-SR Ca gradients and contribute to an even supply of Ca to SR Ca release sites throughout the cardiac myocyte. Furthermore, this would limit local delays in intra-SR Ca movement from Ca uptake to Ca release sites within the SR that could otherwise contribute to reduced SR Ca availability at short diastolic intervals and hence the induction and maintenance of cardiac alternans.

Electron microscopic studies show that the SR is a continuous network with sporadic connections across and along Z-lines.7-9 Wu and Bers10 provided direct functional evidence for continuity of the entire SR and nuclear envelope lumens in adult rabbit cardiac myocytes, using FRAP (fluorescence recovery after photobleach) of an intra-SR Ca indicator (Fluo-5N)11 and Ca movement throughout the SR network. They estimated relatively fast diffusion coefficients for Fluo-5N and Ca within the SR (=10 times slower than free...
diffusion in solution). Another study confirmed the functional SR continuity (using indirect methods), but estimated a 7-fold lower intra-SR Ca diffusion coefficient than ours. Moreover, their computer model suggested that SR Ca release at one junctional SR (JSR) would cause little depletion in neighboring free SR (FSR). Here, we sought to measure spatiotemporal features of FSR versus JSR depletion during local Ca release and assess intra-SR diffusion using local FRAP.

This study was designed to: (1) test whether appreciable JSR-FSR [Ca]SR gradients occur during ECC or local SR Ca release events; (2) test whether intra-SR Ca diffusion on a sarcomeric scale occurs preferentially in the longitudinal versus transverse direction; and (3) examine how spatiotemporal gradients constrain quantitative understanding of SR Ca handling.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Rabbit ventricular myocytes were isolated and loaded with cell-permeant low-affinity Ca indicator Fluo-5N (Kₐ≈400 μmol/L/L) to measure [Ca]SR via confocal microscopy combined with [Ca] measurements using Rhod-2. Standard intact myocyte superfusate (2 mmol/L CaCl₂) and saponin-permeabilized myocyte solutions (150 mmol/L free [Ca]) were used at 21°C to 24°C. Cytosolic and intra-SR FRAP used calcein/acetoxymethylester (AM)- and Fluo-5N/AM-loaded cardiomyocytes, respectively. Data are means±SEM with statistical significance (P<0.05) determined by Student t test.

**Results**

**Limited [Ca]SR Spatial Gradients Accompany ECC**

To test for detectable intra-SR free Ca ([Ca]SR) gradients between Ca release sites (JSR) and nonjunctional free SR sites (FSR) during Ca transients, we used spatially resolved SR Ca depletions during global Ca release in confocal line-scan mode. After steady-state 1 Hz stimulation, the SR structure of Fluo-5N loaded cells was discernible (Figure 1A). JSR is visible as narrow transverse bands of high fluorescence at ≈1.9 μm intervals and FSR appears as faint longitudinal patterns between JSR regions, with variable intensity among FSR regions. Recordings measure fluorescence from intra-SR localized Fluo-5N without appreciable cytosolic signal contamination. JSR is readily distinguishable from the weaker FSR signal (see below).

Figure 1B shows a longitudinal line-scan image (1-Hz stimulation) of 15 JSR and intervening FSR regions. When signals are normalized to diastolic intensity at each location (F/F₀) the localization of individual JSR and FSR regions is no longer apparent during diastole (by definition) or during systole (Figure 1C). The latter indicates that [Ca]SR depletion occurs in FSR (similarly to JSR). [Ca]SR depletions are detectable even at single JSR or FSR sites (Figure 1D, left), and averaging fluorescence (F) of 9 JSR regions over 8 consecutive steady-state twitches illustrates the 50% higher diastolic F at JSR versus FSR (Figure 1D, middle), whereas normalized F/F₀ signals are superimposable (Figure 1D, right). JSR signals are taken from the 1 μm region centered on the intense diastolic JSR signal, whereas FSR signals are taken from the intervening 0.8 to 1 μm that we presume is centered at the M-line at the center of the sarcomere.

JSR and FSR depletion signals were analyzed in 2 different ways. First, a total of 1155 JSR and 753 FSR depletions from 17 cells were aligned at the beginning of the depletion, averaged and normalized (Figure 1E). The time course of JSR and FSR [Ca]SR depletions were very similar, both in amplitude and kinetics. The time to 90% nadir was slightly longer in FSR versus JSR (by 8 ms) suggesting a slight delay between JSR and FSR [Ca]SR depletion during release. Time constants of SR Ca refilling were very similar in JSR and FSR (159 and 140 ms respectively). The average depletion amplitude (∆F/F₀) was 0.30±0.02 for JSR and 0.31±0.03 for FSR, indicating no detectable [Ca]SR gradient at the nadir. Based on previous calibrations, this 30% decrease in Fluo-5N fluorescence corresponds to a 65% decreases in [Ca]SR from ≈1300 to 460 μmol/L.

Next, we analyzed the same SR Ca depletions by fitting individual JSR and FSR depletions with the product of a sigmoid decline and exponential recovery. Kinetic fit parameters were averaged (Figure 1F and 1G). JSR and FSR [Ca]SR depletion amplitudes were similar (0.31±0.03 and 0.32±0.03 ∆F/F₀, respectively). Neither time to nadir of JSR and FSR depletions (101±13 and 88±12 ms, respectively; P=NS) nor the time constant of local [Ca]SR recovery was statistically different (153±24 and 140±26 ms, respectively). These results suggest that intra-SR Ca fluxes are relatively fast without detectable intra-SR [Ca] gradients at the resolution of our experiments. Although there must be some [Ca]SR gradient between JSR release sites and FSR, such differences in kinetics and amplitude must be small. Thus, Ca diffusion is relatively fast within a half-sarcomere, but this does not allow direct assessment of diffusion.

**[Ca]SR Gradients During Spontaneous Local SR Ca Release Events (Blinks)**

Global Ca transients and [Ca]SR scraps, where nearly all junctions release almost simultaneously is the opposite ex-
treme for $[\text{Ca}]_{\text{SR}}$ depletion versus an isolated spontaneous local SR Ca release (Ca spark or blink).\textsuperscript{6} We analyzed spatiotemporal profiles of Ca blinks (measured simultaneously with Ca sparks). Figure 2 shows averaged Ca blinks and sparks during longitudinal (Figure 2A) line-scanning. At the time of local $[\text{Ca}]_{\text{SR}}$ blink nadir ($\approx 40$ ms), the depletion is smaller in amplitude at longer longitudinal distance from the Z-line (50% smaller at a distance of 0.84 $\mu$m from Z-line; Figure 2C). At this same distance the time to nadir is also reached later in time (by $\approx 11$ ms). Thus, there are measurable spatiotemporal $[\text{Ca}]_{\text{SR}}$ gradients during local SR Ca release events.

Transverse line scans gave similar results (Figure 2B). However, the distance for half-maximal depletion was slightly smaller (0.76 versus 0.84 $\mu$m) and the time to nadir occurred later (Figure 2D). Notably, this distance is similar to the transverse JSR spacing.\textsuperscript{16}

This measurable spatiotemporal $[\text{Ca}]_{\text{SR}}$ depletion for Ca blinks, but not for Ca scraps seems dichotomous, but is not.

This is because a single Ca blink can draw from an effectively infinite pool of SR Ca in 3D (and far away there is no $[\text{Ca}]_{\text{SR}}$ depletion). In contrast, when release occurs at each junction simultaneously, each half-sarcomere experiences the same $[\text{Ca}]_{\text{SR}}$ depletion, preventing net diffusion into the domain of one JSR from another. Indeed, each individual half-sarcomere (in 3D) would have reflective boundary conditions (no Ca flux across) and have similar extent of depletion. An alternative way to think about this is from the perspective of one midsarcomere FSR region. This region would only be slightly depleted during a blink at one neighboring Z-line, because that blink can draw Ca from regions in all 3D. For global SR Ca release, that FSR region may be the main reservoir for both adjacent longitudinal JSR regions, and consequently local $[\text{Ca}]_{\text{SR}}$ drops faster and more extensively.

**Variations in Ca Blink Kinetics**

We previously showed that Ca blink kinetics at individual sites vary greatly, but are highly reproducible at a given site.\textsuperscript{6}
This contrasted with the associated Ca sparks which were similar at different loci. Moreover, we reported a correlation between long duration Ca blinks and JSR regions which were highly connected to other SR regions, as assessed by FRAP of intra-SR Fluo-5N. Here, we segregated individual longitudinal Ca blinks from Figure 2 into 2 groups: fast blinks (recovery, t=207 ms; Figure 3A) and slow blinks (t≤200 ms; Figure 3B). Notably, the Ca spark amplitude and kinetics were almost the same for both groups (Figure 3, top middle). This suggests that the amount and kinetics of SR Ca release are roughly comparable. However, the blinks that refilled slowly tended to have slightly longer time to nadir and larger extent of local [Ca\text{\textsubscript{SR}}] depletion. Faster blinks, on the other hand, also exhibited a 28% wider spatial profile (FWHM of depletion was 2.3 versus 1.8 μm; Figure 3, bottom), and the [Ca\text{\textsubscript{SR}}] depletion nadir at 0.8 μm away from the spark site was considerably closer to that seen at the spark center.

These differences are consistent with the fast blinks being from JSR sites that are better connected (via diffusion) to neighboring SR regions than the sites that exhibit slow blinks (JSR sites more diffusionally isolated). That is, SR Ca release at a well connected JSR site causes larger/faster depletion in neighboring SR regions, and recovers faster once SR Ca release terminates (faster t). The slightly deeper nadir in the slow JSR sites may better approximate the critical [Ca\text{\textsubscript{SR}}]\text{\textsubscript{shut-off}} threshold, because slower replenishment allows better temporal separation of release and refilling phases.

**Intra-SR Diffusion: Fluo-5N FRAP**

To directly test intra-SR diffusion, we performed FRAP in cardiomyocytes permeabilized after Fluo-5N SR loading. The confocal line-scan mode for bleaching and FRAP allows high temporal resolution and restricts the bleached region to a width of ≈1.5 μm. Therefore, this protocol allows measurement of intra-SR diffusion on a sarcomeric scale. Scan lines positioned longitudinally (parallel to myocyte long axis) or transversely (perpendicular to long axis at z-line) provides selective measurement of transverse or longitudinal diffusion, respectively.

Figure 4A shows laser intensity profile and Fluo-5N fluorescence from which FRAP kinetics were fit (sum of 2 exponentials). After the 450-ms bleach period, fluorescence decreased by 56±2 and 65±2% (n=25 and 33; P<0.05) of the prebleach intensity for transverse and longitudinal bleach, respectively.

The slow FRAP component was very similar for longitudinal or transverse FRAP (Figure 4B) and constituted ≈60% of the total fluorescence recovery (time constant of ≈36 seconds). The fast FRAP component (~40% of total FRAP), was faster in the longitudinal versus transverse...
direction ($\tau_{fast} = 1.56 \pm 0.2$ s) versus $2.24 \pm 0.2$ s; $P<0.05$; $n=25$ and 33 cells). Thus, intra-SR diffusion is faster longitudinally than transversely. Fluo-5N fluorescence recovered more fully after transverse versus longitudinal bleach (74.8 $\pm$ 2.4 versus 54.4 $\pm$ 1.9%; $P<0.05$, $n=25$ and 33 cells). Incomplete recovery is probably attributable to an immobile fraction of Fluo-5N (protein-bound or mitochondrial trapping) but may also be attributable, in part, to loss of total fluorescent indicator during bleach. The slightly faster longitudinal versus transverse diffusion of Fluo-5N probably reflects geometric SR constraints. Indeed, the FRAP time constants varied in connectivity of individual release units as recently proposed.6,17

We also analyzed single junction FRAP to test for variability in connectivity of individual release units as recently proposed.6-17 Indeed, the FRAP time constants varied $>10$-fold between individual junctions (fits for 10 JSR regions and mean are shown in Figure 4C). The $\tau_{fast}$ varied from 484 ms to 3.8 s (mean $1.57 \pm 0.91$ seconds) and $\tau_{slow}$ varied from 10.2 s to 47.2 s (mean $21.1 \pm 3.5$ seconds). These results are consistent with the notion that the connectivity of individual release units within the SR varies substantially. Junctions that showed very fast FRAP may be comparable with well connected junctions.6

Cytosolic Diffusion: Calcein FRAP

To compare intra-SR with cytosolic diffusion, we used the same FRAP protocol in intact myocytes loaded with the cytosolic fluorophore calcein. Cytosolic FRAP was much faster than within the SR and the kinetics were not different between longitudinal versus transverse bleach. Therefore only longitudinal photobleach is reported ($\tau_{fast} = 0.44 \pm 0.07$ s; 48 $\pm$ 7% of the total recovery and $\tau_{slow} = 7 \pm 1.5$ s; $P<0.05$ versus $\tau_{fast}$). Total FRAP extent was also the same in both directions (59 $\pm$ 4 and 61 $\pm$ 0.2% after longitudinal and transverse photobleach; $n=6$ to 9 cells). Fast (and slow) $\tau$ for cytosolic calcein FRAP was $\approx 4$ to 6 times faster than for Fluo-5N in the SR. The faster calcein FRAP is partly attributable to its smaller molecular weight versus Fluo-5N (622 versus 958), but this would only explain a 24% slower diffusion coefficient for Fluo-5N (Online Table I). Thus, intra-SR diffusion is slower than cytosolic by a factor of $\approx 3$ to 4.

Mathematical Model of [Ca]$_{SR}$ Changes During SR Ca Release

Because these are the first detailed spatiotemporal profiles of dynamic [Ca]$_{SR}$ gradients measured in cardiac myocytes, we created a relatively simple computational model to gain quantitative mechanistic insights. Figure 5A shows the model geometry for global SR Ca release (where all junctions fire). We model a single half-sarcormere and assume that boundary conditions are everywhere reflective (no Ca flux across). For example, longitudinally beyond the sarcormere center (M-line), a mirror image [Ca]$_{SR}$ profile occurs approaching the next SR region. The same holds for sarcosomes above, below, left, and right, as well as across the Z-line. We use our detailed rabbit ventricular myocyte model18 to set overall Ca fluxes (including the SR Ca release flux time course which was initially fixed; see the Online Data Supplement). Ca is released from JSR into the cleft and diffuses along the longitudinal axis both inside and outside the SR (20 sequential compartments), and SR Ca-ATPase is uniformly spread along the nonjunctional SR to allow refilling. We included Ca buffering by calsequestrin only in the 2 JSR compartments.

We simulated global and local Ca transients for different apparent Ca diffusion coefficients ($D_{Ca}^{SR}$; Figure 5C). Figure 5B shows that $D_{Ca}^{SR} = 60 \mu$m$^2$/sec$^{-1}$ (as previously estimated19) produces global and local [Ca]$_{SR}$ signals that resembles those measured experimentally (Figure 1), where there is very little difference in depletion amplitude within the half-sarcormere (<5%) and a relatively short delay (<20 ms). Figure 5C shows the $D_{Ca}^{SR}$ dependence of the $\Delta$[Ca]$_{SR}$ spatial gradient and delay time to nadir between the JSR compartment and...
One at 1 μm (M-line; x = 1 versus 0 μm). To mimic limited optical resolution, we also averaged compartments within 500 nm of the JSR (JSR signal) and of the M-line (FSR signal). If D_Ca^SR were 10 times smaller (6 μm^2/sec^-1),^1^ the Δ[Ca]_{SR} gradient and the delay time would have been easily detectable in Figure 1.

For release from only one junction (Ca blink), we must allow Ca diffusion from neighboring sarcomeres, extending model geometry and boundary conditions (Figure 5D). For simplicity, we included 2 neighboring sarcomeres in each direction from the central releasing junction (20 nearest junctions; see Online Figure II). Further extension adds computational complexity but unaltered results. Using the same SR Ca release flux as in Figure 5A through 5C, substantially greater spatial gradients occur, even at half-sarcomere distance of ~1 μm (Figure 5E). Note that the JSR-FSR [Ca] gradient at nadir is strikingly different for the global versus isolated release, over a broad range of D_Ca (Figure 5F). Moreover, D_Ca^SR = 60 μm^2/sec^-1 produces similar spatiotemporal features as seen in Ca blink experiments in Figure 2. However, for the same driving SR Ca release waveform, the blink [Ca]_{SR} nadir is not as deep (even in JSR) as in a global [Ca]_{SR} depletion. This is unlike experimental data where they are comparable. An obvious explanation is that the blink can refill from all directions (not true for a scrap), which for the same release flux, limits the extent of JSR depletion during a blink. If [Ca]_{SR} decline controls the shutoff of release,^6,^1^ then release ought to continue until the threshold [Ca]_{SR} is reached.

Figure 6 shows simulated Ca blinks where SR Ca release activates, as in Figure 5, but where termination is attributable to an exponential shutoff starting when [Ca]_{SR} reaches 0.46 mmol/L (see the Online Data Supplement). We also use diastolic [Ca]_{SR} = 0.76 mmol/L and average [Ca]_{SR} within 200 nm of the JSR and 0.8 μm compartment, for direct comparison to blinks in Figure 2. Ca spark and blink kinetics roughly match experimental data for D_Ca^SR = 60 μm^2/sec^-1 (Figure 6A, middle). D_Ca^SR greatly influences the kinetics and [Ca]_{SR} gradient (Figure 6A). The [Ca]_{SR} at nadir and the delay in time to nadir over a range of distances from the SR junction (Figure 6E) can be compared to experimental data in Figure 2C and 2D.

Figure 6B shows direct measurement of JSR and FSR (top, from Figure 2), our model with D_Ca^SR = 60 μm^2/sec^-1 (middle) and a model by Swietach et al^1^ using a much lower D_Ca^FSR^ (bottom). Note that the latter model does not capture the FSR Ca depletion measured experimentally.

If transverse and longitudinal D_Ca^SR is the same, the model predicts little anisotropy in [Ca]_{SR} kinetics at 0.8 μm away, but if SR diffusion is faster longitudinally (based on Fluo-5N FRAP data) and we make apparent D_Ca^SR similarly anisotropic, the experimentally observed anisotropy is mimicked (Figure 6C versus Figure 2A and 2B). We can also simulate fast/connected versus slow/isolated junctions by varying D_Ca^FSR (Figure 6D). More sophisticated models are needed, but this simple model is useful to explore spatiotemporal [Ca]_{SR} gradients.

**Discussion**

Our main findings are: (1) during ECC, no appreciable [Ca]_{SR} gradients occur between JSR and FSR; (2) diffusion within the SR is faster in the longitudinal than transverse direction (unlike cytosolic diffusion which is isotropic); (3) spatiotemporal [Ca]_{SR} gradients are measurable during isolated Ca blinks; (4) intra-SR diffusion of Fluo-5N is faster than a similar sized fluorophore in the cytosol; and (5) intra-SR Ca diffusion varies locally, dependent on local SR connectivity. These results have important implications for understanding the dynamics of SR Ca handling and [Ca]_{SR} homogeneity, which may be critical for normal synchrony of myocyte and heart contraction and arrhythmogenesis.

**[Ca]_{SR} Gradients Between SR Ca Release and Uptake Sites**

Analyzing a large number of Ca scraps (spatially resolved [Ca]_{SR} decline during global SR Ca release) JSR-FSR [Ca]_{SR} gradients or delays were undetectable. Of course, there must be some [Ca]_{SR} gradient and delay between the site of release and sites distant from release, but at the level of an individual sarcomere this appears to be small (<5%), brief (<10 ms), and difficult to detect. This places explicit constraints on models of Ca diffusion and intra-SR buffering. Wu and Bers^10^ showed that the lumen of the entire SR network and nuclear
envelope are continuous. On activation of SR Ca release during ECC, nuclear envelope [Ca] was depleted to only $\approx 60\%$ of that for sarcomeric SR, and nuclear envelope [Ca] kinetics were delayed with respect to [Ca]$_{SR}$ (time to nadir was 50 ms longer and recovery time constant was 70 ms longer). So, over longer distances, transient intra-SR [Ca] gradients are readily detected. Other reports also suggested [Ca]$_{SR}$ gradients during local SR Ca release from individual junctions (Ca blinks),$^6,9,19$ where neighboring junctions do not release simultaneously (as in Ca scraps$^{11}$).

These are the first spatiotemporally detailed measurements of [Ca]$_{SR}$ during Ca sparks/blinks. During local Ca blinks fractional [Ca]$_{SR}$ depletion falls to approximately half the JSR value at $\approx 0.8 \mu m$ away (ie, approximately midsarcomere), and [Ca]$_{SR}$ depletion is negligible at the next longitudinal JSR region 2 $\mu m$ away. There is also temporal delay in blink time to nadir with increasing distance from JSR. This indicates that these gradients are technically measurable, supporting the conclusion that [Ca]$_{SR}$ gradients are small when all junctions release simultaneously. The average [Ca]$_{SR}$ nadir for both local Ca blinks and global Ca scraps during ECC are very similar ($\Delta F/F_0 \approx 0.3$, or [Ca]$_{SR} \approx 400 \mu mol/L$).

The similar [Ca]$_{SR}$ nadir for JSR depletion during both Ca blinks and global Ca release (scraps) is consistent with SR Ca release termination occurring at a particular JSR [Ca]$_{SR}$ threshold.$^3,6$ During a global Ca scrap, each junction may shut off at that same local [Ca]$_{SR}$, but at that point, the FSR between these sites has also reached this same level. Notably, when Ca scraps occur at every junction during ECC, a given FSR region will be depleted by at least the 2 nearest JSR regions (plus those in parallel sarcomeres). Our measurements of Ca scraps and blinks place novel quantitative constraints for SR Ca handling during ECC.

These observations agree with Ca blinks being fundamental local release events that add to compose global Ca scrap signals (as Ca sparks are to Ca transients), and that most of the Ca involved in a release event normally comes from the region within $\approx 1 \mu m$ of the junction (yielding little spatial summation). The latter differs from Ca sparks, where extensive spatiotemporal summation of Ca sparks results in larger and slower [Ca] transients (attributable to spatiotemporal overlap).$^6$ The adequacy of local SR Ca to support JSR release is aided by JSR Ca buffering by calsequestrin, and allows SR Ca release to suffice for contractile activation.
without emptying the SR. Our analysis also suggests that the SR Ca release waveform during a blink may be more prolonged (versus scraps), because local JSR takes longer to decrease to the release termination threshold \([\text{Ca}]_{\text{SR}}\) when there is a larger diffusional reserve.

**Intra-SR Ca Diffusion Coefficients**

Wu and Bers\(^{10}\) used direct \([\text{Ca}]_{\text{SR}}\) measurements with Fluo-5N to measure intra-SR Ca diffusion and an apparent diffusion coefficient (D\(_{\text{Ca-SR}}\)) of \(\approx 60 \mu \text{m}^2/\text{sec}\) (\(\approx 10\) times slower than in solution), but substantially faster than cytosolic D\(_{\text{Ca}}\) in muscle (which is reduced by Ca binding to fixed binding sites; \(\approx 14 \mu \text{m}^2/\text{sec}\)).\(^{20}\) Swietach et al\(^{12}\) estimated a slower D\(_{\text{Ca-SR}}\) in cardiac myocytes (8 to 9 \(\mu \text{m}^2/\text{cm}\)), using an indirect approach (cytosolic Ca changes in response to local caffeine application). These are apparent diffusion coefficients, which include effects of local Ca buffering and path tortuosity. In the cytosol, fixed high-affinity Ca binding sites (like calsequestrin) have low affinity and rapid off-rates, and would hinder diffusion less. On the other hand, the SR network, while highly interconnected, follows a tortuous path, such that path tortuosity might be a larger constraint on Ca diffusion inside the SR. Indeed, fluorophore diffusion inside the SR is 3 to 4 times slower than in cytosol, suggesting that about half of the 10-fold slowing of Ca diffusion in SR is attributable to pathway tortuosity, whereas the rest may be attributable to relatively fixed buffers inside SR.\(^{12}\)

We cannot explain why Swietach et al\(^{12}\) estimated a much smaller D\(_{\text{Ca-SR}}\) than Wu and Bers\(^{10}\) or that we find here. However, their explanation, that Wu and Bers did not account for SR Ca leak and declining [Ca\(_{\text{SR}}\)] during measurements is unfounded because direct [Ca\(_{\text{SR}}\)] measurements were shown (Figure 7C in the article by Wu and Bers\(^{10}\)), which exclude that explanation. However, their explanation, that Wu and Bers did not account for SR Ca leak and declining [Ca\(_{\text{SR}}\)] during measurements is unfounded because direct [Ca\(_{\text{SR}}\)] measurements were shown (Figure 7C in the article by Wu and Bers\(^{10}\)), which exclude that explanation. Moreover, the elegant model by Swietach et al\(^{12}\) (using indirect D\(_{\text{Ca-SR}}\) estimates) fails to recapitulate FSR depletion during Ca blinks that we measure experimentally (Figure 6B). The precise definition of D\(_{\text{Ca-SR}}\) in each report also differs slightly.
Regardless of absolute $D_0$, our direct $[Ca]_{SR}$ measurements during ECC indicate that intra-SR Ca diffusion is fast enough to nearly abolish potential $[Ca]_{SR}$ gradients between JSR and FSR within our spatiotemporal resolution. Fast intra-SR diffusion is further evident from the results of our FRAP experiments, showing that even the high molecular weight molecule Fluo-5N can diffuse rapidly within the SR network. Our prior work estimated intra-SR diffusion coefficients for Fluo-5N and Ca that differed only by the amount expected for their difference in molecular weight.\textsuperscript{10} This is most consistent with a situation where the main diffusional limitation is tortuosity (which would be the same for both) rather than binding to fixed buffers (which ought to differ). Here, cytosolic calcein in the cytosol and Fluo-5N in the SR show that Fluo-5N diffusion inside SR is $\approx 3$ to 4 times slower than in cytosol. This may reflect largely differences in tortuosity, but other factors (eg, binding) cannot be excluded.

Ca flux rate depends not only on $D_0$, but also on the [Ca] gradient ($\Delta C$) and cross-sectional area ($A$; $J_{Ca}=D_0 A (\Delta C/\Delta x)$, where $x$ is longitudinal distance). Even for the same $D_0$, in cytosol and SR, higher $\Delta [Ca]$ in SR than cytosol greatly enhance longitudinal SR Ca flux rate. For a 2-fold concentration gradient $\Delta C$ would be $\approx 500 \text{ mmol/L}$ in SR versus $<0.5 \text{ mmol/L}$ in cytosol).

**Consequences of Relatively Fast Intra-SR Ca Diffusion**

Relatively fast intra-SR Ca diffusion has several functional consequences. First, it creates homogenous SR Ca driving force for release during ECC (preventing regions with low $[Ca]_{SR}$). It may also limit spontaneous SR Ca release (preventing regions with high $[Ca]_{SR}$). Furthermore, there is no major temporal delay between $[Ca]_{SR}$ recovery in the JSR and FSR (as implicit in some earlier models). If Ca diffusion is faster in SR than cytosol, it could limit Ca wave propagation. That is, if depletion at one junction reduces $[Ca]_{SR}$ at the next junction, it reduces the likelihood that elevated $[Ca]$ will activate the downstream release site (because of $[Ca]_{SR}$ effects on ryanodine receptor gating). A single Ca spark may not decrease $[Ca]_{SR}$ appreciably at the next longitudinal junction $2 \text{ mm away}$ (Figure 2C), but this may not be so true transversally where distance between JSR regions is only $\approx 0.8 \text{ mm}$ or after a wave is already initiated (ie, where multiple depleting events from the wave direction could reduce local JSR $[Ca]$ ahead of the wave).

Data regarding local SR Ca and wave propagation are indirect and controversial. Acute SERCA blockade was reported to either accelerate or slow Ca waves.\textsuperscript{5,22} One could argue that blocking SERCA either keeps local cytosolic [Ca] higher in the wavefront, thereby enhancing propagation (preventing reuptake along the way), or that Ca reuptake along the wave drives $[Ca]_{SR}$ up ahead of the wave such that blocking uptake slows propagation by limiting $[Ca]_{SR}$. SERCA activity could have both of these competing effects (explored theoretically\textsuperscript{23,24}). Direct $[Ca]_{SR}$ measurements during waves would help to resolve this apparent dichotomy.

**Intra-SR Diffusion Is Slightly Anisotropic and Junctions Vary in Connectivity**

For cytosolic diffusion FRAP was the same longitudinally and transversely (as in homogeneous cell-free systems). Thus, cytosolic calcein diffusion is relatively isotropic, despite highly organized myocyte structures, which differ longitudinally and transversely. In contrast, intra-SR FRAP was faster in the longitudinal versus transverse direction. This must mean that longitudinal SR connections (eg, around or between T-tubules) create less of a diffusional barrier than the transverse connections (eg, between sarcomeres in parallel). Ca blink characteristics also suggest a faster longitudinal versus transverse intra-SR Ca diffusion. That is, $[Ca]_{SR}$ depletion $0.8 \text{ mm away}$ is smaller for transverse versus longitudinal Ca blinks (Figure 2A versus 2B) and the delays in time to nadir are longer in the transverse direction (Figure 2D). The functional consequence is not obvious, but it could contribute to differential transverse wave susceptibility as discussed above). The smaller transverse depletion might also be attributable, in part, to a closer reservoir of the Ca buffer calzequin, because of the greater proximity to neighboring JSR regions in the transverse direction. This intra-SR anisotropy differs from cytosolic Ca diffusion, which appears isotropic because Ca sparks are symmetrical in longitudinal and transverse directions.\textsuperscript{25} For wave propagation, a transverse junction would see higher cytosolic [Ca] (because it is closer than the nearest longitudinal junction), and $[Ca]_{SR}$ may be relatively maintained (because of weaker transverse SR diffusion). Both of these effects would enhance transverse versus longitudinal wave propagation.

FRAP analysis revealed large variation in individual junctions even within the same cell. This is consistent with recent work\textsuperscript{4,17} showing that some SR Ca release units are much better connected within the SR network than others. This may not matter during physiological ECC, because $[Ca]_{SR}$ gradients are tiny and refilling depends primarily on SR Ca-ATPase. However, during Ca sparks/waves, filling is predominantly diffusion-dependent,\textsuperscript{6} and there could be heterogeneous refilling with some regions reaching threshold for release before others. That could contribute to arrhythmogenic substrate. Despite differential predominance of SR Ca-ATPase versus diffusion, both global Ca scraps and local Ca blinks have similar recovery $\tau$ (150 to 200 ms). This is probably because SR Ca-ATPase is much faster during the larger global Ca transient and can compensate for the diffusional component present only during the blink. Both our experimental data and mathematical model provide novel understanding of spatiotemporal dynamics of $[Ca]_{SR}$ and place novel constraints on how we view $[Ca]_{SR}$.

**Sources of Funding**

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is Known?**

- Free intra-sarcoplasmic reticulum (SR) \([\text{Ca}^2+]_{\text{SR}}\) \(([\text{Ca}^2+]_{\text{SR}})_{\text{SR}}\) is a critical factor in regulating the strength of the heartbeat, the normal termination of SR Ca2+ release, and the initiation and propagation of arrhythmogenic Ca2+ waves; however, there are limited direct measurements of \([\text{Ca}^2+]_{\text{SR}}\).

- The SR network is connected throughout the myocyte, and the lumen of the SR also communicates with the nuclear envelope.

**What New Information Does This Article Contribute?**

- \([\text{Ca}^2+]_{\text{SR}}\) was imaged during normal cardiac myocyte contractions (synchronous release from all sites) and during spontaneous Ca2+ sparks or blinks when only local Ca2+ release occurs.

- Ca2+ diffuses rapidly within the SR network. During normal Ca2+ transients there are only very small \([\text{Ca}^2+]_{\text{SR}}\) gradients between the nonfunctional and junctional SR regions.

- During spontaneous local Ca2+ release, large gradients of \([\text{Ca}^2+]_{\text{SR}}\) develop, creating nonuniformity that could impact arrhythmogenicity.

- Observations quantitatively describe intra-SR Ca2+ regulation, permitting the development of a mathematical model of SR Ca movements.

- Compared with the cytosol, diffusion within the SR is 3 to 4 times slower and is faster in the longitudinal rather than the transverse direction.

- \([\text{Ca}^2+]_{\text{SR}}\) is a critically important regulator of both the normal cardiac excitation–contraction coupling (ECC) process and spontaneous SR Ca release. It provides the driving force for the Ca2+ release that activates contraction, as well as potentially arrhythmogenic ionic currents. \([\text{Ca}^2+]_{\text{SR}}\) is also a critical allosteric regulator of the sensitivity of the SR Ca2+ release channel to activation by Ca2+–induced Ca2+ release, the probability of initiation of arrhythmogenic spontaneous Ca2+ sparks and waves, and the termination of SR Ca2+ release during both ECC and Ca2+ sparks. However, little direct data are available on the real dynamics of intra-SR Ca2+ movements. Here, we measure the \([\text{Ca}^2+]_{\text{SR}}\) directly in live myocytes with high spatiotemporal resolution. Our study provides key novel quantitative insights into exactly how \([\text{Ca}^2+]_{\text{SR}}\) changes locally during normal ECC and spontaneous Ca2+ release events. Moreover, we provide a framework for understanding the dynamics of \([\text{Ca}^2+]_{\text{SR}}\) in cardiac myocytes similar to the models currently available for cytosolic \([\text{Ca}^2+]\). These detailed aspects of SR Ca2+ depletion have both physiological and pathophysiological implications for the heart during heart failure and triggered arrhythmias.
Dynamic Calcium Movement Inside Cardiac Sarcoplasmic Reticulum During Release
Eckard Picht, Aleksey V. Zima, Thomas R. Shannon, Alexis M. Duncan, Lothar A. Blatter and Donald M. Bers

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SUPPLEMENTAL MATERIAL
for
Dynamic Calcium Movement in Cardiac Sarcoplasmic Reticulum during Release

Eckard Picht, Aleksey V. Zima, Thomas R. Shannon, Alexis M. Duncan, Lothar A. Blatter, Donald M. Bers

METHODS

Cell isolation

Single ventricular myocytes were isolated from New Zealand White rabbits using standard enzymatic techniques. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and approved by the Institutional Animal Care and Use Committee. Animals were anaesthetized with sodium pentobarbital (50 mg/kg i.v.). Hearts were excised, rinsed in cold Ca-free solution, and mounted on a Langendorff perfusion system. Hearts were perfused at 37°C with Ca-free solution (DMEM, Dulbecco’s minimal essential medium, Gibco/Invitrogen, USA, gassed with 95% O2-5% CO2) for 5 min. Solution was then switched to a collagenase (0.3-0.7 mg/ml; Boehringer Mannheim, Germany) and Ca (10-20 μM) containing solution. After 15–25 min, perfusion was stopped and ventricles were minced into small pieces. Optionally the pieces were incubated for 5–20 min in fresh enzyme. Finally, enzyme activity was stopped with DMEM solution containing bovine serum albumin (BSA; 0.5-1%), and tissue was agitated or triturated to liberate single myocytes. Cells were washed and stored in DMEM solution adjusted to [Ca] = 150 μM. All experiments were performed at room temperature (21-24°C).

Fluo-5N loading and confocal imaging of [Ca]SR

Isolated cardiomyocytes were loaded with the cell-permeable form of the low affinity Ca indicator Fluo-5N (Kd ~400 μM/L) as described previously.1,2 In short, cells were incubated for 2 hours with 15 μM Fluo-5N/AM at 37°C, and 1.5 hours was allowed for deesterification and outward leak of cytosolic dye. Cells were washed and stored in DMEM solution adjusted to [Ca] = 150 μM. All experiments were performed at room temperature (21-24°C).

Ca spark and blink measurements

For simultaneous recording of Ca sparks and corresponding [Ca]SR depletions (Ca blinks) we used the high affinity Ca indicator Rhod-2 and the low affinity Ca indicator Fluo-5N, respectively. Fluo-5N/AM loaded myocytes were permeabilized with saponin (0.005% for 30 s) as previously described.4 The saponin free internal solution contained (in mmol/L unless indicated): K aspartate 100; KCl 15; KH2PO4 5; MgATP 5; EGTA 0.35; CaCl2 0.12; MgCl2 0.75; phosphocreatine 10; HEPES 10; Rhod-2 tripotassium salt 0.04; creatine phosphokinase 5 U/ml; dextran (MW: 40,000) 8%, and pH 7.2 (KOH). Free [Ca] and [Mg] of this solution were 150 nmol/L and 1 mmol/L, respectively.

Changes in cytosolic [Ca] and [Ca]SR were measured simultaneously with a laser scanning confocal microscope (Radiance 2000 MP, Bio-Rad, UK) equipped with a 40× oil-immersion objective lens (N.A.=1.3). Fluo-5N was excited with the 488 nm laser line of an argon ion laser and fluorescence was measured at wavelengths > 500 nm. The linescan mode was used at a scanning speed of 166 lines per second with the scanning line parallel to either the longitudinal or transverse myocyte axis at a central focal plane (avoiding the nucleus). Cells were field-stimulated at 1 Hz using platinum electrodes until steady-state was achieved. Caffeine (10 mmol/L) was applied at the end of the experiment to determine the Ca insensitive fraction of the Fluo-5N fluorescence that was subsequently used to correct the raw fluorescence traces. [Ca]SR depletions were fitted with the product of a sigmoid decline and exponential recovery phase2 and analyzed offline using custom-made software (Detector V2.3, E.P). With this approach, and care to only use myocytes with monophasic decreases in Fluo-5N fluorescence during excitation-contraction coupling1-3 the Fluo-5N signals can be taken as a direct indicator of free [Ca]SR.
SparkMaster. For each detected Ca spark the corresponding Ca blink was analyzed as described previously. The profiles of Ca blinks were fit as the product of a rising and decaying exponential function. Amplitudes of sparks and blinks are expressed as F/F₀, where F₀ is the initial fluorescence before release. All Ca blinks were corrected for the caffeine-insensitive component of Fluo-5N fluorescence after complete SR Ca depletion with 10 mmol/L caffeine. For [Ca]₀ calibrations, Fₘᵦᵣᵣ and Fₘₐₓ was assessed in each myocyte, and the apparent Kₛ(Ca) was assumed to be 400 µM. Fₘᵦᵣᵣ was measured after depletion of the SR with 10 mmol/L caffeine in the presence of 5 mmol/L EGTA. Fₘₐₓ was measured following an increase of [Ca] to 10 mmol/L. Caffeine (10 mmol/L) keeps RyRs open allowing [Ca] equilibration across the SR membrane. To prevent irreversible cell contraction during application of high [Ca] cells were pretreated for 5 min with the muscle contraction uncouplers 2,3-butanedione monoxime (10 mmol/L) and blebbistatin (10 µmol/L).

### FRAP experiments

Cytosolic and intra-SR FRAP experiments were performed in line scan mode (488 nm excitation) at a scanning speed of 166 lines per second. Cytosolic FRAP experiments were performed on cells which were loaded with the cell permeable form of the Ca-independent fluorescent dye Calcein. Myocytes were incubated with 5 µM of Calcein/AM for 10 min followed by 30 min of superfusion with normal Tyrode’s solution to allow deesterification. Intra-SR FRAP experiments were performed in Fluo-5N loaded cardiomyocytes after cell permeabilization with saponin as described before. Permeabilization washes out cytosolic indicator; however, some Fluo-5N may be trapped in mitochondria, but because intra-mitochondrial [Ca] is low at the 150 nM [Ca] used here’ mitochondrial Fluo-5N will only weakly fluoresce. Moreover, because the mitochondria are isolated, they cannot participate in the FRAP kinetics measured (i.e. they could only contribute to the lack of complete FRAP extent).

Confocal line-scan mode photobleach was performed, restricting the area of photobleaching to the width of a sarcomere while allowing a high temporal resolution FRAP measurements. By bleaching either along the longitudinal or transverse direction of the myocyte, we selectively investigated intra-SR diffusion in the transverse or longitudinal direction of the SR (i.e. perpendicular to the direction of the bleach). By bleaching transversely along a z-line, FRAP occurs via diffusion perpendicular to the bleached line (i.e. due to diffusion in the longitudinal direction) because no concentration gradients exist along the direction of the bleach.

The FRAP protocol consisted of a 72 ms pre-bleach scan at 10% laserpower to determine the baseline fluorescence. This was followed by a photobleaching period lasting 450 ms at 75% laser power followed by 12 post-bleach scans of 72 ms duration immediately following the photobleach and distributed over 75 s. The laserpower of the short scans after the photobleaching during FRAP was the same as used for the pre-bleach scan. In between the recovery scans the laserpower was set to 0% to minimize photobleaching during the FRAP measurement. The recovery kinetics were fit with the sum of two exponentials.

All chemicals were from Sigma-Aldrich (St. Louis, MO). Fluo-5N/AM and Rhod-2 were purchased from Molecular Probes/Invitrogen (Carlsbad, CA).

### Statistics

Data are presented as means±SEM of n measurements. Statistical comparisons between groups were performed with the Student t test. Differences were considered statistically significant a P<0.05.

### Diffusion of Fluo-5N and Calcein during FRAP

For FRAP measurements we used Fluo-5N to assess intra-SR diffusion and calcein to assess cytosolic diffusion. These differ in molecular weight (MW) and so would be expected to differ in their apparent diffusion coefficient (see Table I). Correcting the relative D value for MW allows better comparison among the FRAP data. The MW-adjusted τ-fast for Fluo-5N FRAP suggests that diffusion within the SR is 3-4-fold slower (depending on direction) than in the cytosol. This would be consistent with greater tortuosity (or possibly viscosity) inside SR vs. cytosol.

### Table I. Molecular weights and FRAP kinetics corrected for size

<table>
<thead>
<tr>
<th></th>
<th>MW(g/mol)</th>
<th>(MW)⁰·⁵</th>
<th>Relative D*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluo-5N</td>
<td>958</td>
<td>30.95</td>
<td>1</td>
</tr>
<tr>
<td>Calcein</td>
<td>622</td>
<td>24.94</td>
<td>1.24</td>
</tr>
</tbody>
</table>

*Relative D theoretically depends inversely on molecular weight (MW)⁰·⁵

<table>
<thead>
<tr>
<th></th>
<th>τ slow (s)</th>
<th>τ-fast (s)</th>
<th>τ-fast (s)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-SR Fluo-5N</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>longitudinal</td>
<td>36</td>
<td>1.56</td>
<td>1.56</td>
</tr>
<tr>
<td>Transverse</td>
<td>36</td>
<td>2.24</td>
<td>2.24</td>
</tr>
<tr>
<td>Cytosolic Calcein</td>
<td>7</td>
<td>0.44</td>
<td>0.55</td>
</tr>
</tbody>
</table>

*Adjusted for molecular weight (MW)

### Mathematical Model

#### General Information

All programming was done using Matlab (The Mathworks, Inc., Natick, MA, United States). The model is composed of a system of differential equations describing changes in [Ca] within SR and cytosolic compartments separated by the SR membrane. Ca was
conserved such that the sum of the Ca in the SR and the cytosol was held constant. The program was typically run for 5 seconds to assure steady-state had been achieved before simulating a release event.

Diffusion of all molecules (S) was described by a special case of Fick’s Law:

$$\frac{d[S]}{dt} = D_S \frac{d^2[S]}{dx^2}$$

where $D_S$ is the diffusion coefficient (Table II). All compartments were spaced at a distance, $dx$, of 50 nm. No attempt was made to distinguish the rate of diffusion of Ca bound and unbound ligands.

**Table II: Diffusion Coefficients**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Diffusion coefficient ($\mu$m$^2$/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR Ca</td>
<td>As indicated</td>
</tr>
<tr>
<td>Fluo 5N</td>
<td>8</td>
</tr>
<tr>
<td>Cytosolic Ca</td>
<td>240</td>
</tr>
<tr>
<td>Rhod-2</td>
<td>42</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>0.5</td>
</tr>
<tr>
<td>ATP</td>
<td>0.14</td>
</tr>
<tr>
<td>EGTA</td>
<td>0.036</td>
</tr>
</tbody>
</table>

Ca binding to buffering substances within the cell was described by the rate equation:

$$\frac{d[Ca\cdot L]}{dt} = k_{on}[Ca](B_{max}[Ca\cdot L]) - k_{off}[Ca\cdot L]$$

where $L$ is the buffering ligand (Table III). For additional details concerning buffering and kinetics see Shannon et al.\(^9\) and Bers.\(^10\) The initial condition was set by calculating the amount of ligand bound using the Hill equation:

$$[Ca\cdot L] = B_{max}[Ca]/([Ca]+K_d)$$

**Table III: Ca Buffering Parameters**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>$B_{max}$ (umol/l)</th>
<th>$k_{on}$ (µM s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$K_d$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troponin C Ca</td>
<td>70</td>
<td>32.7</td>
<td>19.6</td>
<td>0.6</td>
</tr>
<tr>
<td>Ca-Mg (Ca)</td>
<td>140</td>
<td>2.37</td>
<td>0.033</td>
<td>0.0135</td>
</tr>
<tr>
<td>Ca-Mg (Mg)</td>
<td>140</td>
<td>0.003</td>
<td>3.33</td>
<td>1111</td>
</tr>
<tr>
<td>Calmodulin</td>
<td>24</td>
<td>34</td>
<td>238</td>
<td>7</td>
</tr>
<tr>
<td>Myosin</td>
<td>140</td>
<td>13.8</td>
<td>0.46</td>
<td>0.0333</td>
</tr>
<tr>
<td>Myosin (Mg)</td>
<td>140</td>
<td>0.0157</td>
<td>0.057</td>
<td>3.64</td>
</tr>
<tr>
<td>SR</td>
<td>19</td>
<td>100</td>
<td>60</td>
<td>0.6</td>
</tr>
<tr>
<td>SL</td>
<td>42</td>
<td>100</td>
<td>1300</td>
<td>13</td>
</tr>
<tr>
<td>SL (hi affinity)</td>
<td>15</td>
<td>100</td>
<td>30</td>
<td>0.3</td>
</tr>
<tr>
<td>Fluo-4/ Rhod-2</td>
<td>25</td>
<td>100</td>
<td>110</td>
<td>1.1</td>
</tr>
<tr>
<td>ATP$^*$</td>
<td>5000</td>
<td>150</td>
<td>3000</td>
<td>200</td>
</tr>
<tr>
<td>ATP (Mg)$^*$</td>
<td>5000</td>
<td>1.95</td>
<td>19.5</td>
<td>100</td>
</tr>
<tr>
<td>EGTA$^*$</td>
<td>350</td>
<td>12.5</td>
<td>2</td>
<td>0.16</td>
</tr>
<tr>
<td>Calsequestrin</td>
<td>140</td>
<td>100</td>
<td>65000</td>
<td>650</td>
</tr>
</tbody>
</table>

$^*$Only present in permeabilized myocytes

Ca fluxes were driven by Ca release from the SR which was represented by the waveform based on measurements in Shannon et al.\(^11\)\(^,\)\(^12\) (see below). Released Ca was re-sequestered back into the SR by SR Ca pumps which operated as simple reversible enzymes:

$$J_{pump} = V_{max}([Ca]/K_{mf})^{-1}$$

$$([Ca]/K_{mf})^{-1}/(1+[Ca]/K_{mf})^{-1}$$

where the parameters are as in Table IV.

**Table IV: SR Ca Pump Parameters**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$</td>
<td>137 µM/s</td>
</tr>
<tr>
<td>$K_{mf}$</td>
<td>0.26 µM</td>
</tr>
<tr>
<td>$K_{mr}$</td>
<td>1.82 mM</td>
</tr>
<tr>
<td>$H$</td>
<td>2</td>
</tr>
</tbody>
</table>

**Whole Cell Model**

Whole cell Ca release was simulated in a half sarcomere with reflective boundaries (under the simplifying assumption that all junctions release during normal excitation-contraction coupling\(^13\)\(^,\)\(^14\) The half-sarcomere consists of both SR and cytosolic compartments, each 50 nm in length along the long axis of the sarcomere. Longitudinally the half-sarcomere consisted of 20 SR compartments along with 20 corresponding cytosolic compartments. SR compartments one and two were considered to be junctional space containing calsequestrin. When displaying JSR signals that are averages of several compartments (to simulate optical measurements) the first 2 junctional compartments received five times greater weighting than the surrounding SR compartments to mimic the higher Fluo-5N concentration in these locations. Release was from compartment one into the corresponding cytosolic compartment, considered to be adjacent to the sarcolemma. Released Ca therefore diffused from this compartment through the adjacent compartments toward the center of the sarcomere. Ca was re-sequestered back into the SR through SR Ca pumps located in compartments 2-20 and diffused back towards compartment one through the SR.

![Image](https://example.com/image.png)

**Figure 1.** Kinetics of SR Ca release flux for A) the case of global E-C coupling and the first case for single junction release (in Fig 5D-F of the manuscript) and B) for single junction release where release shuts off when local [Ca]$^{2+}$ declines to 40% of the diastolic level (as in Fig 6 of the manuscript). Faster diffusion coefficients delay the time to shut off.
Transversely along the Z-line the half sarcomere consisted of two sets of 10 SR compartments, each meeting the longitudinal SR at compartment two. These compartments were considered to be passive conduits for Ca, without cytosolic compartments or SR Ca-ATPase associated with them.

The release waveform was determined from that described by Shannon et al.11 (see Fig I). To simulate measured kinetics, we used an empirical product of three Boltzman equations:

\[
J_{\text{Release}} = \frac{(1.43 \times 10^{-5} + (8.5 - 1.43 \times 10^{-5})/(1 + 10^{20.54 - t} \times 0.3976))}{(12 + (0.008749 - 12)/(1 + 10^{0.7139} \times 0.0124))}(75 + (0.25 - 75)/(1 + 10^{2.037 - t} \times 0.1672))
\]

Where t is time in ms and \( J_{\text{Release}} \) is in mmol/l cytosol/s.

**Release from Single Junctions**

For a single junction release (Ca spark/blink) we cannot use the same reflective boundaries, because Ca can diffuse from the SR in regions more than a half-sarcomere away (and of course cytosolic Ca can likewise diffuse away from the junction to neighboring regions). Here we used the geometry shown in Fig II, where the center (red) junction is the active junction that is diffusionally connected (intra-SR and cytosol) to two sarcomeres in each orthogonal direction (including one diagonal junction in each direction. The figure only shows two dimensions (x-y), but we included the same geometry in the vertical (z) direction (i.e. rotating Fig II 90° around the longitudinal axis). Thus we are including the 20 nearest junctions from one central release unit. We added another layer further out from this, but it did not appreciably alter the results.

Two Ca release waveforms were used. The first was identical to that for whole cell release. The second release form consisted of a rising exponential followed by a decaying exponential (see Fig IB). The rising exponential was defined as

\[
J_{\text{Release}} = 2(1-e^{-0.0135(t-t_0)})
\]

This release continued until a 40% decline in [Ca]_{SR} in the releasing junction was reached at which point release terminated with an exponential decline as:

\[
J_{\text{Release}} = J_0(e^{-0.0135(t-t_0)})
\]

where \( J_0 \) is the flux and t0 is the time (in ms) at which the threshold [Ca]_{SR} is reached.

**References**