Sarcoplasmic Reticulum and Nuclear Envelope Are One Highly Interconnected Ca\(^{2+}\) Store Throughout Cardiac Myocyte

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Abstract—Previous ventricular myocyte studies indicated that ryanodine receptors (RyRs) are in the sarcoplasmic reticulum (SR) and are critical in excitation–contraction coupling, whereas the inositol trisphosphate (InsP\(_3\)) receptors are separately localized on the nuclear envelope (NucEn) and involved in nuclear Ca\(^{2+}\) signaling. Here, we find that both caffeine and InsP\(_3\) receptor agonists deplete free [Ca\(^{2+}\)] inside both SR and NucEn. Fluorescence recovery after photobleach (FRAP) was measured using the low-affinity Ca\(^{2+}\) indicator Fluo-5N trapped inside the SR and NucEn (where its fluorescence is high because [Ca\(^{2+}\)] is \(\approx 1\) mmol/L). After Fluo-5N photobleach in one end of the cell, FRAP occurred, accompanied by fluorescence decline in the unbleached end with similar time constants (\(\tau \approx 2\) minutes) until fluorescence regained spatial uniformity. Notably, SR and NucEn fluorescence recovered simultaneously in the bleached end. Ca\(^{2+}\) diffusion inside the SR-NucEn was also measured. SR Ca\(^{2+}\)-ATPase was completely blocked but without acute SR Ca\(^{2+}\) depletion. Then caffeine was applied locally to one end of the myocyte. In the caffeine-exposed end, free SR [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{sa}\)) declined abruptly and recovered partially (\(\tau = 20\) to 30 seconds). In the noncaffeine end, [Ca\(^{2+}\)]\(_{sa}\) gradually declined with a similar \(\tau\), until [Ca\(^{2+}\)]\(_{sa}\) throughout the cell equalized. We conclude that the SR and NucEn lumen are extensively interconnected throughout the myocyte. Apparent intrastore diffusion coefficients of Fluo-5N and Ca\(^{2+}\) were estimated (\(\approx 8\) \(\mu\)m\(^2\) sec\(^{-1}\) and 60 \(\mu\)m\(^2\) sec\(^{-1}\)). This rapid luminal communication may maintain homogeneously high luminal [Ca\(^{2+}\)] for local Ca\(^{2+}\) release events from either SR or NucEn. (Circ Res. 2006;99:283-291.)

Key Words: sarcoplasmic reticulum ■ nuclear envelope (NucEn) ■ fluorescence recovery after photobleach (FRAP) ■ caffeine

Cardiac myocytes respond to extracellular stimuli by different signaling pathways. An important mechanism in cardiac myocytes is excitation–contraction coupling (ECC).\(^1\) Electrical depolarization of the cell membrane opens L-type Ca\(^{2+}\) channels, causing Ca\(^{2+}\) influx. This Ca\(^{2+}\) entry activates ryanodine receptors (RyRs) on the intracellular Ca\(^{2+}\) store sarcoplasmic reticulum (SR), inducing robust SR Ca\(^{2+}\) release. This local Ca\(^{2+}\) -induced Ca\(^{2+}\) release (CICR)\(^2\) is the essence of ECC.\(^3\)

Intracellular Ca\(^{2+}\) stores include SR (endoplasmic reticulum [ER] in nonmuscle cells) and nuclear envelope (NucEn). Free [Ca\(^{2+}\)] inside these stores can be \(\approx 1\) mmol/L, which is similar to that in the extracellular space.\(^3\) The SR is composed of junctional SR (jSR), which is covered by RyRs and faces toward the T tubules, and free SR (fSR), which contains SR-ER Ca\(^{2+}\)-ATPase (SERCA).\(^4\) The NucEn surrounds the nucleus and has both an inner and an outer membrane and a space in between where [Ca\(^{2+}\)] can be millimolar.

Recently, we found that cardiac myocytes use local Ca\(^{2+}\) release from inositol trisphosphate (InsP\(_3\)) receptors (InsP\(_3\)R) in the NucEn to respond to the neurohumoral stimuli in excitation–transcription coupling (ETC).\(^5\) Endothelin-1 (ET-1) application caused InsP\(_3\) to activate local Ca\(^{2+}\) release from the NucEn via InsP\(_3\)R, which activated calmodulin and CaMKII to phosphorylate histone deacetylase-5 (causing its nuclear export) and activation of transcription.\(^5\) Notably, this pathway could only be activated by Ca\(^{2+}\) from local InsP\(_3\)R (not global Ca\(^{2+}\) transients), presumably because calmodulin and CaMKII physically associate with the InsP\(_3\)R at the NucEn.\(^5\) Indeed, in ventricular myocytes, RyRs are mainly in SR, whereas InsP\(_3\)R are mainly in NucEn.\(^6\) Thus, SR and NucEn differ structurally and functionally and may reflect discrete physically different Ca\(^{2+}\) stores. In addition, the relatively rigid sarcomeric organization in striated muscle with a dense protein mesh at the Z-lines raises the possibility that local SR within a given sarcomere could serve its particular sarcomere but not necessarily communicate with neighboring sarcomeres either longitudinally or transversely. The main aim of the present study was to determine whether different SR regions and NucEn constitute discrete separate Ca\(^{2+}\) stores.
In some cell types (nonmuscle cells), it is generally accepted that the outer nuclear membrane and ER have similar characteristics and outer nuclear membrane is continuous with ER membrane including SERCA, whereas the inner nuclear membrane is quite different from the outer nuclear membrane. However, it is not known whether the lumen of ER/SR and NucEn is subcompartmentalized or continuous. The existence of separated Ca\(^{2+}\) stores has been proposed by some groups, but rapid diffusion of Ca\(^{2+}\) and other molecules between the lumen of NucEn and ER was also shown in leukemia cells and fibroblasts.

Here we assess diffusion inside the SR and NucEn using the low-affinity Ca\(^{2+}\) indicator Fluo-5N, fluorescence recovery after photobleach (FRAP) and local caffeine applications. We found evidence that the SR and NucEn are highly interconnected and comprise 1 large continuous intracellular Ca\(^{2+}\) store. This will help to understand ECC and ETC, as well as cardiac arrhythmias, because inhomogeneous SR Ca\(^{2+}\) release may contribute to Ca\(^{2+}\) waves.

Materials and Methods

Myocyte Isolation Fluo-5N Loading and Cell Permeabilization

Myocytes were isolated as previously described and loaded with Fluo-5N acetoxymethylester (10 \(\mu\)mol/L) for 2 hours and deesterified for 1.5 hour. For intact myocytes, the superfusate contained (in mmol/L): NaCl 140, KCl 4, MgCl\(_2\) 1, CaCl\(_2\) 2, HEPES 10, glucose 10 (pH 7.4, 23°C). For permeabilization, myocytes were exposed to relaxing solution (in mmol/L: EGTA 0.1, HEPES 10, K-aspartate 120, free MgCl\(_2\) 1, ATP 5, reduced glutathione 10, phosphocreatine di-Tris 5 [pH 7.4]) and then permeabilized by saponin (50 g/mL) for 20 seconds. After permeabilization, we used an internal solution (same as relaxing solution, but free Ca\(^{2+}\)) was adjusted to 100 nmol/L and 8% dextran was added to prevent swelling on permeabilization, with pH 7.2. [Ca\(^{2+}\)]\(_{\text{NucEn}}\) was calculated using pseudo-ide method described previously for single-wavelength indicator. F0 corresponds to resting [Ca\(^{2+}\)]\(_{\text{NucEn}}\), which was taken to be 1 nmol/L.

Imaging Under Confocal Microscopy

Images were acquired with a Bio-Rad Confocal Microscope (488 nm argon laser excitation, emission at 500 nm long pass). Laser power was 100% for photobleach, 4% for line scan images, and 0.5% for whole cell images. Because the laser power for image recording is low, unintended photobleach was negligible. Confocal imaging used a x40 oil immersion objective and temporal resolution of 166 lines per second. ImageJ software was used for image analysis.

For some FRAP experiments, the pinhole diameter was increased to 12 mm (maximal) to obtain fluorescence from the whole cell thickness. However, in caffeine experiments (where release occurs at all depths) and other experiments, pinhole diameter was chosen for confocality (1.2 mm). All fluorescence data were background subtracted.

Simulation of Fluo-5N Diffusion Inside the Ca\(^{2+}\) Store

The cell/ER-NucEn was considered a circular cylinder, with cross-section area \(A\) and length \(L\), divided into 20 equal-volume compartments (\(\Delta L = L/20\)). Diffusion was allowed between neighboring compartments (boundary flux was set to 0). For each compartment, Fluo-5N fluorescence or [Ca\(^{2+}\)]\(_{\text{Ca\(^{2+}\)}}\) (C) was determined by Fluo-5N or Ca\(^{2+}\) diffusion from/to the adjacent compartments. The initial curve was from experimental data fitted to sigmoid curve. The change in \(C\) in compartment \(n\) per unit time (\(\Delta C/\Delta t\)) is

\[
\Delta C/\Delta t = D(\frac{C_{n-1} - C_n}{\delta - C_{n+1}})/\Delta L V_n
\]

where \(D\) is the apparent diffusion coefficient for Fluo-5N or Ca\(^{2+}\), \(V_n\) is the volume of compartment \(n\), and \(V_n = A\Delta L\). The above applies to the cylindrical cell structure. SR inside the cell has different structure and accounts for only 3% to 5% of the cell volume. The SR cross-section area should also be only 3% to 5% of that of the whole cell. However, because \(V_n = A\Delta L\), equation 1 can be independent of \(A\).

\[
\Delta C/\Delta t = D([C_{n-1} - C_n] - [C_{n+1} - C_n])/(\Delta L V_n)
\]

Thus, \(D\) is determined by concentration gradients and \(\Delta L/\Delta t\). Note that \(D\) (as defined here) will be reduced by contributions of binding of Ca\(^{2+}\) or Fluo-5N inside the SR-NucEn and also tortuosity of diffusional path (ie, noncylindrical actual geometry of the network). At a time point \(t\), \(C\) in compartment \(n\) (\(C_n\)) follows:

\[
C_n(t) = C_{n-1} + \Delta C_n(t)
\]

Equation 2 was solved numerically with \(\Delta t = 0.1\) second. The value of \(D\) was varied to obtain a least square fit to the measured spatiotemporal \(C_n\) profiles from experiments.

Statistical Analysis

Data are presented as mean±SEM with significance (\(P<0.05\)) determined using unpaired 2-tailed Student \(t\) test.

Results

Caffeine or InsP\(_3\)R Agonists Can Deplete [Ca\(^{2+}\)]\(_{\text{SR}}\) in Both SR and NucEn

Rabbit ventricular myocytes were loaded with Fluo-5N and permeabilized with saponin. Fluo-5N is a low-affinity Ca\(^{2+}\) indicator (\(K_i = 400 \mu\)mol/L), only bright where [Ca\(^{2+}\)] is very high, as in SR and NucEn (Figure 1A). We also see irregularly distributed bright spots which are caffeine and InsP\(_3\)-insensitive (most obvious in Figure 6). The Fluo-5N signal was stable when myocytes were left in skinned fiber solution without any treatment (Figure 1A). This shows that [Ca\(^{2+}\)] inside SR and NucEn are stable and that no appreciable Fluo-5N is lost over the experimental time course. Caffeine (10 mmol/L) application causes an immediate decline in Fluo-5N signal both in the SR and NucEn (Figure 1B and 1C), with the SR preceding the NucEn. Figure 1D shows SR and NucEn [Ca\(^{2+}\)]\(_{\text{SR}}\) depletions during electrically evoked Ca transients (0.5 Hz). Again, [Ca\(^{2+}\)]\(_{\text{SR}}\) declined transiently (Ca\(^{2+}\) scaps) in both SR and NucEn. However, in the NucEn, the depletion amplitude was smaller and delayed in both time to nadir and recovery time constant (\(r_{\text{rec}}\)). These results are consistent with RyR-dependent intra-SR Ca depletions driving those in the NucEn, but they do not prove that the stores are connected. On the other hand, if RyRs are only on the SR (not the NucEn), and if the SR and NucEn are not connected, then caffeine and twitchs should not cause NucEn Ca\(^{2+}\) depletion.

Application of InsP\(_1\) (10 \(\mu\)mol/L) or the potent InsP\(_3\)R agonist adenophostin (10 \(\mu\)mol/L) for 30 minutes also gradually decreased the Fluo-5N signal in both NucEn and SR in permeabilized myocytes (Figure 2A and 2B and time course of Fluo-5N signal changes in Figure 2C). Note that InsP\(_3\)R activation produced a very much slower less complete Ca\(^{2+}\) depletion of the SR and NucEn. This is consistent with the much lower number of InsP\(_3\)R (versus RyR) in ventricular myocytes (and lower intrinsic Ca\(^{2+}\) flux rate).

That is, the very high Ca\(^{2+}\) flux rate via caffeine-activated RyRs completely depletes [Ca\(^{2+}\)]\(_{\text{SR}}\) (despite continued SERCA function). In contrast, the much lower InsP\(_3\)R flux rate only slowly depletes [Ca\(^{2+}\)]\(_{\text{SR}}\) and results in a new steady state where the
SERCA pump and leak rates rebalance at a new low $[\text{Ca}^{2+}]_{\text{SR}}$. This explains why the lack of time delay between SR and NucEn signals (similar $\tau$ values), because the InsP$_3$-induced leak rate may be small compared with potentially rapid NucEn-SR $\text{Ca}^{2+}$ equilibration. Again, if InsP$_3$R are only on the NucEn (not SR) and the 2 pools are not interconnected, this result is unexpected. This raises the possibility that the SR and NucEn lumens are highly connected. However, the above phenomena could also happen even if the SR and NucEn were not connected but if there were very small numbers of RyRs on the NucEn and of InsP$_3$R on the SR. These could be functionally effective even if their existence is only near the detection limit of immunochemistry, fractionation methods, or Western blot analyses. To test whether the lumen of the SR is connected to both the NucEn and distant regions of the SR, we performed the following FRAP and caffeine-induced $[\text{Ca}^{2+}]_{\text{SR}}$ depletion experiments.

**Fluo-5N Diffusion Inside the SR and NucEn:**

**FRAP Studies**

Permeabilized, Fluo-5N–loaded myocytes were photo-bleached on approximately half of the cell longitudinally with the other half left almost unaffected (Figure 3A). Because of dispersion and scatter of illumination, the beam was centered near the left cell end, causing an initial gradient of bleach that was maximal at the end of the cell but gradually declining to be negligible at the cell center (Figure 3A and 3B). Fluorescence in the bleached half (left) decreased immediately on bleach. After bleach, the SR and NucEn fluorescence increased gradually in the bleached half, and simultaneously there was a complementary gradual decrease of fluorescence in the unbleached half (right) until the 2 halves reached the same level (full FRAP; Figure 3A, with longitudinal profile in Figure 3B). On Fluo-5N photobleach, local $[\text{Ca}^{2+}]_{\text{IS}}$ gradients are neither likely to be significant nor to complicate subsequent FRAP measurements. This is partly because the intrastore Fluo-5N concentration is very low compared with physiological SR $\text{Ca}^{2+}$ buffers (eg, calsequestrin; see Figure II in the online data supplement, available at http://circres.ahajournals.org). Rather, subsequent FRAP is attributable...
almost entirely to the diffusion of Fluo-5N within the SR and NucEn. Note that in these and subsequent longitudinal profiles, the regular sarcomeric pattern of jSR (with 1.9-μm spacing3; see Figure 3B inset) was intentionally eliminated by smoothing.

Figure 3C shows the time course of FRAP in the bleached half and the decay of fluorescence in the unbleached half. The total fluorescence after bleach (~15% overall bleach) did not change during FRAP, indicating that these measurements are not complicated by changes in [Ca2+]SR. The time constant for FRAP on the left and fluorescence decline on the right were similar (τ=112.1±9.8 s and 115.3±5.7 seconds). The NucEn on the left also demonstrated FRAP, with similar kinetics as the SR (τ=119.8±7.6 seconds).

This FRAP experiment was also done in the transverse direction (Figure 4). In this image, the upper side of the cell was bleached and transverse FRAP and complementary fluorescence decline in the bottom occurred. The results are qualitatively similar for transverse versus longitudinal FRAP, except that the time constants were faster transversely (~15 seconds) than longitudinally (~110 seconds). This is not surprising because the distance is much less in the transverse direction. Both longitudinal and transverse FRAP were complete and spatially uniform. Because these were permeabilized myocytes and Fluo-5N was trapped inside the SR and NucEn, the recovery of the bleached half can only be attributable to the Fluo-5N diffusion inside the Ca2+ store. In Figure 3A and 3C, both NucEn and SR on the left recover during FRAP, evidence of extensive connection of the SR with both the NucEn and with more distant SR regions.

Because the images in Figures 3 and 4 were confocal, the recovery of fluorescence on the bleached half could be partially attributed to Fluo-5N diffusion from regions above and below the confocal plane (in addition to the other half in the x-y plane). On the other hand, bleach in the Z direction is likely to be substantial (as it is nonconfocal). Nevertheless, to avoid FRAP from above and below the confocal plane, the signal from the whole thickness of the cell was obtained by opening the confocal pinhole maximally (12 mm versus 1.2 mm in confocal images). As in Figure 3, we bleached the left half of the cell (including 1 nucleus), whereas the right half remained relatively unaffected (Figure 5A).
are fuzzier because of intentional nonconfocality. The fluorescence on the left half dropped immediately by 30% (Figure 5B). The degree of photobleach was only 30% because of the brief laser exposure (less than 1 second to limit cell damage and optimize temporal resolution). FRAP is apparent in both SR and NucEn on the bleached half, accompanied by a gradual and complementary decline on the unbleached half. We compared FRAP kinetics by choosing region of interest (ROI). The \( \tau \) values for the recovery of both halves were similar, \( \approx 2 \) minutes (Figure 5B). The amplitudes of the post-bleach decrease and increase of fluorescence in the 2 halves were also about the same. The post-bleach total cell fluorescence was constant (Figure 5B). Again, because this was in a permeabilized cell and the only source of Flu-5N was from the SR and NucEn, this result means that Flu-5N diffuses from one end of the cell to the other inside the SR-NucEn network. If Flu-5N came out of the cell, it would diffuse away and we would see loss of total fluorescence. This indicates that the \( \text{Ca}^{2+} \) stores of the 2 halves are connected.

At the same longitudinal location, the bleach and recovery of the SR and NucEn were also compared for different ROI (Figure 5C). The SR and NucEn regions were bleached to the same extent and recovered with a similar time constant. Because the recovery of NucEn can only be via Flu-5N diffusion from the SR, this demonstrates that the SR and NucEn are extensively connected. A region of SR closer to the bleached end of the cell had more extensive bleach (60% versus 30% on average for this half) and also recovered with a slightly longer \( \tau \) (see simulations below). The similar FRAP results in Figures 3 and 5 confirm control experiments, indicating photobleach through most of the cell depth.

**Ca\(^{2+}\) Diffusion Inside the SR and NucEn**

We also performed caffeine-induced \( \text{[Ca}^{2+}]_{\text{SR}} \) depletion at one end of the cell to assess \( \text{Ca}^{2+} \) diffusion inside the SR and NucEn. This was done in intact cells under special conditions, previously developed and validated, where SERCA could be completely blocked but SR stores maintained until a release is triggered. Intact myocytes were paced at 0.5 Hz for 10 beats to preload \( \text{Ca}^{2+} \) stores. Then pacing was stopped and perfusion was switched for 90 seconds to a \( \text{Na}^{+} \)-free, \( \text{Ca}^{2+} \)-free NT (\( \text{Li}^{+} \) replaced \( \text{Na}^{+} \) and 10 mmol/L EGTA was included) with 5 \( \mu \text{mol/L} \) thapsigargin (TG) (to block the SERCA pump). Using this protocol, \( \text{[Ca}^{2+}]_{\text{SR}} \) was not decreased significantly (Figure 6A and 6B). However, SERCA was completely blocked because, after acute \( \text{[Ca}^{2+}]_{\text{SR}} \) depletion by caffeine (Figure 6C), caffeine washout and then field stimulation \( \text{[Ca}^{2+}]_{\text{SR}} \) could not be detectably refilled (Figure 6D versus 6C). After treatment with TG, the SR \( \text{Ca}^{2+} \) content does eventually decline (even in 0 \( \text{Na}^{+} \)/0 \( \text{Ca}^{2+} \) solution), but there is a practical time window (\( \approx 5 \) minutes) in which these experiments can be done. Note that the caffeine-insensitive bright spots were also unaffected throughout the procedure.

In cells pretreated with TG 0 \( \text{Na}^{+} \)/0 \( \text{Ca}^{2+} \), as above, we evaluated intra-SR \( \text{Ca}^{2+} \) diffusion using local caffeine application. Figure 7A shows local caffeine application at the left end of the cell briefly (1 to 2 seconds) to release \( \text{Ca}^{2+} \) from SR and NucEn at this end. There is an acute discernable \( \text{[Ca}^{2+}]_{\text{SR}} \) gradient from the left end to the middle (Figure 7A and 7B). Because SERCA was completely blocked, the only source of \( \text{Ca}^{2+} \) recovery at the left end is \( \text{Ca}^{2+} \) diffusion within the \( \text{Ca}^{2+} \) store from the right. If this recovery happens, SR and NucEn must be connected throughout the myocyte. Indeed, \( \text{[Ca}^{2+}]_{\text{SR}} \) recovery at the left and right ends occurred with similar \( \tau \) values (Figure 7C). Additionally, the total \( \text{[Ca}^{2+}]_{\text{SR}} \) inside the \( \text{Ca}^{2+} \) store was constant, indicating acute SR \( \text{Ca}^{2+} \) release, no net reuptake and no further loss (Figure 7C). In addition, the recovery of SR and NucEn occur simultaneously with similar \( \tau \) values (Figure 7D). This again implies that SR and NucEn are highly connected via their lumens.

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**Figure 5.** Fluo-5N FRAP in permeabilized myocyte (whole cell image). A, Laser (100%) was used to bleach the left half (including 1 nucleus). Images were acquired at times indicated after photobleach (pinhole=12 mm). B, Time course of average fluorescence (F) in each half of myocyte (outlined by dashed lines in A, 0-second image, N=7 cells). C, FRAP for 3 ROI (illustrated in A, 60-second image; N=6 cells).
The SR and NucEn Are Highly Interconnected in Cardiac Ventricular Myocytes

The data here measuring both Fluo-5N FRAP and Ca\textsuperscript{2+} diffusion inside the SR, directly demonstrate for the first time that the SR lumen is highly interconnected with the NucEn and also with distant regions of the SR. Moreover, the SR-NucEn throughout the myocyte appears to be a single large continuous Ca\textsuperscript{2+} store.

Discussion

The SR and NucEn Are Highly Interconnected in Cardiac Ventricular Myocytes

The data here measuring both Fluo-5N FRAP and Ca\textsuperscript{2+} diffusion inside the SR, directly demonstrate for the first time that the SR lumen is highly interconnected with the NucEn and also with distant regions of the SR. Moreover, the SR-NucEn throughout the myocyte appears to be a single large continuous Ca\textsuperscript{2+} storage compartment. Our initial expectation was that the SR within each sarcomere would be relatively isolated from other SR regions. On the other hand, occasional electron micrographs show that jSR can connect SR from 1 side of the Z-line to the other (and to transverse sarcosomes as well), but there has been no prior functional data to indicate the extent to which the SR network is continuous. Clearly it is. Similarly, there have been reports that the outer nuclear membrane is continuous with the ER in some cell types, but no prior functional evidence in cardiac myocytes. Indeed, the apparent difference in Ca\textsuperscript{2+} release channels (RyR in SR and InsP\textsubscript{3}R in NucEn) and functional roles (ECC for SR and ETC for NucEn) might lead one to expect that the pools are separate. Clearly this is not the case. These processes and release channels are fueled by the same Ca\textsuperscript{2+} pool. We were surprised how rapid diffusion appears to be within the SR-NucEn network.

Intra–SR-NucEn Diffusion

In our simple simulation, the estimated values for D\textsubscript{Ca\textsuperscript{2+}}\textsubscript{Store} and D\textsubscript{Fluo\textsuperscript{x}} were 60 \(\mu\text{m}^2\text{sec}^{-1}\) and 8 \(\mu\text{m}^2\text{sec}^{-1}\), respectively (ie, 7.5 times faster for Ca\textsuperscript{2+}). Because D is inversely related to the square root of molecular weight, the expected ratio is 5.3 times higher D for Ca\textsuperscript{2+} versus Fluo-5N. Given the many factors that could complicate our simplistic analysis, this is in rather good agreement. The estimated D\textsubscript{Ca\textsuperscript{2+}}\textsubscript{Store} is approximately 10-fold slower than expected in aqueous solution (700 \(\mu\text{m}^2\text{sec}^{-1}\)). Main factors that would reduce D\textsubscript{Ca\textsuperscript{2+}}\textsubscript{Store} to fixed sites, high viscosity, and tortuosity of the diffusion path. We favor tortuosity as a dominant factor because: (1) both D\textsubscript{Ca\textsuperscript{2+}}\textsubscript{Store} and D\textsubscript{Fluo\textsuperscript{x}} seem reduced roughly in parallel; (2) path-length tortuosity is expected and would affect both similarly; and (3) because intra-SR buffering of Ca\textsuperscript{2+} and Fluo-5N are likely very different. Indeed, images of the SR network structure would suggest substantial tortuosity. Of course Ca\textsuperscript{2+} also binds to intra-SR buffers like calsequestrin, but the low-affinity (K\textsubscript{d} = 0.5 mmol/L) and high off-rate would limit the impact on D\textsubscript{Ca\textsuperscript{2+}}\textsubscript{Store}. Notably, our D\textsubscript{Ca\textsuperscript{2+}}\textsubscript{Store} of 60 \(\mu\text{m}^2\text{sec}^{-1}\) is much faster than the apparent D\textsubscript{Ca\textsuperscript{2+}}\textsubscript{Store} estimated in myoplasm (1.4 \(\mu\text{m}^2\text{sec}^{-1}\)). Whereas tortuosity and viscosity may contribute to the low apparent D\textsubscript{Ca\textsuperscript{2+}} in cytosol, Ca\textsuperscript{2+} binds to many, relatively high-affinity (slow off-rate) fixed Ca\textsuperscript{2+} binding sites in the cytosol (eg, troponin C, SERCA, calmodulin, myosin). Thus, Ca\textsuperscript{2+} binding may be the major limitation for Ca\textsuperscript{2+} diffusion in myoplasm, versus tortuosity inside the SR. The faster Ca\textsuperscript{2+} diffusion inside the SR may also move more Ca\textsuperscript{2+} because [Ca\textsuperscript{2+}]\textsubscript{SR} is 10 000 times higher than in cytosol. Fast intra-SR Ca\textsuperscript{2+} diffusion may also be important to consider in analyzing propagating waves of CICR in muscle and other cell types.

Ca\textsuperscript{2+} Wave Initiation and Propagation

Ca\textsuperscript{2+} waves are thought to initiate when [Ca\textsuperscript{2+}]\textsubscript{SR} is elevated (perhaps locally), and this initiates a local SR Ca\textsuperscript{2+} release event (Ca\textsuperscript{2+} spark). The wave then propagates via CICR, as released Ca\textsuperscript{2+} diffuses to the next junction where it triggers local SR Ca\textsuperscript{2+} release and so on. Note that local [Ca\textsuperscript{2+}]\textsubscript{SR} at the next site must be sufficiently high for RyRs to be activated by the lower local [Ca\textsuperscript{2+}], (<1 mmol/L) than occurs via local
Ca\textsuperscript{2+} current during normal ECC (>10 μmol/L).\textsuperscript{2} The rapid equilibration within the SR-NucEn described here would help to keep 1 area from experiencing a higher local [Ca\textsuperscript{2+}]\textsubscript{SR} (ie, the network buffers local [Ca\textsuperscript{2+}]\textsubscript{SR} elevation and depletion), thus potentially limiting initiating events for diastolic Ca\textsuperscript{2+} waves. It may also interfere with wave propagation, if local [Ca\textsuperscript{2+}]\textsubscript{SR} ahead of the initiating wave could decrease (because of release at the previous region) before release at that next junction is activated by high local [Ca\textsuperscript{2+}]. That is, lower local [Ca\textsuperscript{2+}]\textsubscript{SR} could desensitize local RyRs at the next site. Both of these effects might decrease the likelihood of wave initiation and propagation. Of course, these ideas need future experimental tests.

Local elevation of [Ca\textsuperscript{2+}]\textsubscript{SR} was suggested as a potential mechanism of arrhythmogenesis in hypertrophic heart,\textsuperscript{15} causing loci prone to wave initiating. Given the present results, this scenario seems unlikely, unless the SR is more fragmented in hypertrophy or heart failure. In preliminary experiments in a rabbit heart failure model,\textsuperscript{24} we did not uncover any fragmentation of the SR-NucEn using the same FRAP approach used here (not shown). This may merit further exploration in pathophysiological models.

**Local SR Ca\textsuperscript{2+} Depletion During SR Ca\textsuperscript{2+} Release**

Rapid Ca\textsuperscript{2+} diffusion within the SR (along with buffering by calsequestrin) can enhance SR Ca\textsuperscript{2+} release through the jSR by minimizing the local SR Ca\textsuperscript{2+} depletion, which occurs during ECC. Indeed, the first direct measurements of [Ca\textsuperscript{2+}]\textsubscript{SR} and Ca\textsuperscript{2+} scraps during normal ECC showed that there was no detectible delay in the time course of local [Ca\textsuperscript{2+}]\textsubscript{SR} decline in fSR versus jSR.\textsuperscript{3} This has been confirmed in subsequent measurements,\textsuperscript{25} consistent with rapid intra-SR diffusion within a sarcomere. Local photolytically induced CICR was shown to have an extremely short refractoriness compared with global ECC,\textsuperscript{26} and this was ascribed to very rapid replenishment of local SR Ca\textsuperscript{2+} release sites by neighboring SR regions. Brochet et al\textsuperscript{25} observed local isolated [Ca\textsuperscript{2+}]\textsubscript{SR} depletion during Ca\textsuperscript{2+} sparks (called Ca\textsuperscript{2+} blinks). They took the detectability of these events as evidence of restricted fSR-jSR diffusion. However, both the extent of [Ca\textsuperscript{2+}]\textsubscript{SR} depletion and recovery time constant were much smaller for Ca\textsuperscript{2+} blinks versus Ca\textsuperscript{2+} scraps or global SR Ca\textsuperscript{2+} release. These results, we think are consistent with very rapid Ca\textsuperscript{2+} replenishment from neighboring SR regions as we show here throughout the whole cell.

Our measurements here are over a spatial scale that makes [Ca\textsuperscript{2+}]\textsubscript{SR} gradients readily detectable. This places a lower limit on D\textsuperscript{fSR} (60 μm sec\textsuperscript{-1}) within a single sarcomere. We scaled down our simple 1D model to a half-sarcomere. We decreased [Ca\textsuperscript{2+}]\textsubscript{SR} instantaneously from 1 to 0.5 mmol/L only in the junctional 50-nm compartment (corresponding to a maximal local jSR release). With our D\textsuperscript{fSR} value of 60 μm\textsuperscript{2} sec\textsuperscript{-1}, the [Ca\textsuperscript{2+}]\textsubscript{SR} gradient throughout that 1-μm half-sarcomere within 2 ms is less than 3% (0.96 to 0.99 mmol/L) versus 25% if we had used D\textsubscript{Ca} reported for myoplasm (1.4 μm\textsuperscript{2} sec\textsuperscript{-1}; see supplemental Figure I). These results are...
consistent with the lack of detectable jSR-fSR \([Ca^{2+}]_{SR}\) gradient during Ca\(^{2+}\) scraps. This simple model is a starting point for further considerations.

**Uniform Ca\(^{2+}\) Distribution Is Physiologically Significant**

The spatially uniform \([Ca^{2+}]_{SR}\) provides a spatially uniform driving force for SR Ca\(^{2+}\) release throughout the myocyte during ECC. This may be important in producing homogeneous Ca\(^{2+}\) release and contractile activation throughout the ventricular myocyte. The network also ensures fidelity of local SR or NucEn Ca\(^{2+}\) release events involved in local Ca\(^{2+}\) signaling. That is, the entire network of SERCA pumps in the SR-NucEn help maintain this \([Ca^{2+}]_{SR}\) gradient for all its uses. The results in Figure 2 show that InsP\(_{3}\) or adenophostin reduces \([Ca^{2+}]_{SR}\) everywhere, even if the primary functional targets and locus of release are at the NucEn. The incomplete \([Ca^{2+}]_{SR}\) depletion indicates that the leak generated by strong InsP\(_{3}\)R activation is less than the overall forward SERCA pumping rate at 100 nmol/L \([Ca^{2+}]_{i}\) (\(\approx 20 \mu\)mol/L cytosol per second) or more than 100 times slower than SR Ca\(^{2+}\) release via RyR during a twitch or caffeine-induced Ca\(^{2+}\) transient). A potential cost of this rapid equilibration is that any pathophysiological SR Ca\(^{2+}\) leak could deplete \([Ca^{2+}]_{SR}\) everywhere in the cell and limit both RyR and InsP\(_{3}\)R-mediated Ca\(^{2+}\) release.

In conclusion, the cardiac SR is highly interconnected to both NucEn and more distant SR throughout the entire myocyte. Fast Ca\(^{2+}\) diffusion within this SR-NucEn store may

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**Figure 8.** Simulation of Fluo-5N and Ca\(^{2+}\) diffusion inside the Ca\(^{2+}\) store. A, Schematic of one dimensional diffusion model (see Materials and Methods). B, Longitudinal profile of Fluo-5N diffusion raw data (fitted to sigmoid curve) at each compartment for 4 exemplar time points (of 8 analyzed). C, Simulated curves corresponding to those in B. D, Theoretical curves for time course of Fluo-5N recovery at indicated distances from left end of myocyte. The inset shows \(r\) values at different distances. E, Longitudinal profile of Ca\(^{2+}\) diffusion data (fitted to sigmoid curve) at each compartment and time point. F, Simulations for representative curves corresponding to those in E. G, Theoretical kinetics of \([Ca^{2+}]_{SR}\) recovery after local caffeine application for different longitudinal locations (inset shows \(r\) values at different distances for \([Ca^{2+}]_{SR}\) recovery).
stabilize local free $[\text{Ca}^{2+}]_{\text{lm}}$ to provide uniform driving force for RyR and InsP$_3$. Homogeneous $[\text{Ca}^{2+}]_{\text{lm}}$ may ensure uniform and synchronous SR Ca$^{2+}$ release and contractile activation.

Acknowledgments
We thank Drs Eckard Picht and Fei Wang for helpful discussions and Jayme O’Brien and Karl Hench for technical assistance.

Sources of Funding
Supported by NIH grants HL30077 and HL64724 (D.M.B.) and an American Heart Association Fellowship (to X.W.).

Disclosures
None.

References
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_Circ Res._ 2006;99:283-291; originally published online June 22, 2006;
doi: 10.1161/01.RES.0000233386.02708.72

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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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:
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**METHODS**

Myocytes were isolated as previously described. \(^{16}\) All procedures were performed in accordance with *Guide for the care and Use of Laboratory Animals* and approved by the Institutional Animal Care and Use Committee. Briefly, adult rabbits were anaesthetized by intravenous (IV) injection of 4.6 ml Nembutal with anticoagulant heparin 1.4 ml. After bilateral thoracotomy, hearts was excised quickly and mounted on a Langendorff perfusion apparatus, perfused for 5-7 min with nominally Ca\(^{2+}\) free Dulbecco's Modified Eagle's Medium (DMEM) solution (perfusion pressure =60-80 mmHg). Perfusion was then switched to the same solution containing 1 mg/ml collagenase until the heart became flaccid. Ventricles were then dispersed and filtered.

**RESULTS**

Figure S1 (below) shows the simple diffusional model described in the Methods and Fig 7, scaled down to the length of a half-sarcomere (1 µm). Here we simulate a sudden and maximal drop (50%) in [Ca\(^{2+}\)]\(_{\text{SR}}\) localized in the junctional SR (L=0) and examine how rapidly diffusion within the half-sarcomere dissipates that [Ca\(^{2+}\)]\(_{\text{SR}}\) gradient. We assume that the other half-sarcomere (from L = 1 µm to 2 µm) behaves similarly in a mirror image (reflected across the M-line) and that the next sarcomere to the left (L = –1 µm to 0) is similarly reflected across the z-line. The neighboring parallel sarcomeres would also be like the modeled one. Thus whether or not SR regions are connected (longitudinally, transversely or both), the boundary conditions are such that the half-sarcomere model is unaltered (assuming normal synchrony among SR Ca\(^{2+}\) release sites).

We used the apparent diffusion coefficients D\(_{\text{Ca}}\)\(_{\text{Store}}\) estimated from the measurements of Ca\(^{2+}\) diffusion inside the SR (60 µm\(^2\)s\(^{-1}\); Panel A) and that previously estimated in myoplasm\(^1\) (1.4 µm\(^2\)s\(^{-1}\); Panel B). For our estimated D\(_{\text{Ca}}\)\(_{\text{Store}}\) the sarcomeric [Ca\(^{2+}\)]\(_{\text{SR}}\) gradient dissipates from 50% to 3% in less than 2 ms and less than 1% in 4 ms. Thus in 4 ms, [Ca\(^{2+}\)]\(_{\text{SR}}\) is essentially uniform between jSR and fSR.

In contrast, the sarcomeric gradient in Panel B is still very large (25% at 2 ms) and a 10% difference in [Ca\(^{2+}\)]\(_{\text{SR}}\) is still present for 15 ms. The faster D\(_{\text{Ca}}\)\(_{\text{Store}}\) (60 µm\(^2\)s\(^{-1}\)) is consistent with results where during a normal SR Ca\(^{2+}\) release there is no detectable [Ca\(^{2+}\)]\(_{\text{SR}}\) spatial gradient between the jSR and fSR. \(^{2,3}\)
**Figure S2:** We measured the concentration of Fluo-5N inside the SR and NucEn by observing fluorescence of known [Fluo-5N] on the confocal stage in vitro with internal solution (mM: EGTA 0.5, HEPES 10, K-aspartate 120, MgCl₂ 1 free, ATP 5, reduced glutathione 10, phosphocreatine di-tris 5, free [Ca²⁺] 1, 8% dextran, BSA 50 mg/ml, pH 7.2) mimicking the intrastore environment (and approximate Fluo-5N saturation level). There, is a linear relationship between the [Fluo-5N] (salt form was used since this is an *in vitro* measurement) and at the identical confocal settings we measured 2-D confocal images of Fluo-5N loaded cells (where [Ca]₅₆ is expected to be ~ 1 mM). The average fluorescence of myocyte image is 40-60 units (A.U.). This corresponds to 2.5-5 μM [Fluo-5N] (see the dashed lines in Figure S2). However, the SR and NucEn volume is only occupy ~5% of total cell volume. Thus the real [Fluo-5N] in the store must be about (2.5-5)/0.05 = 50-100 μM. This is very small compared with other physiological Ca²⁺ intra-SR Ca²⁺ buffers like calsequestrin, which has 3-10 mM Ca²⁺ binding sites in the SR (with comparable Ca²⁺ affinity to Fluo-5N).

In our experiments, we photobleached 30-40% of the Fluo-5N. This could release only up to 10-28 μM Ca²⁺ (<1% of calsequestrin buffering) even if fluorophores bleach destroys Ca²⁺ binding. According to Molecular Probes, bleaching the Fluo-5N fluorophores does not decrease its Ca²⁺ binding. Thus [Ca²⁺]₅₆ is probably not appreciably changed by the bleach. This also means that Ca²⁺ diffusion between the bleached and unbleached part is likely to be an insignificant complication. Moreover, photobleach affects both the Ca²⁺-free and bound forms of Fluo-5N (which we confirmed), so the balance between bound and unbound should not change either.

**References**