**Ca\(^{2+}\) Scraps**

**Local Depletions of Free [Ca\(^{2+}\)] in Cardiac Sarcoplasmic Reticulum During Contractions Leave Substantial Ca\(^{2+}\) Reserve**

Thomas R. Shannon, Tao Guo, Donald M. Bers

**Abstract**—Free [Ca\(^{2+}\)] inside the sarcoplasmic reticulum ([Ca\(^{2+}\)]\(_{\text{SR}}\)) is difficult to measure yet critically important in controlling many cellular systems. In cardiac myocytes, [Ca\(^{2+}\)]\(_{\text{SR}}\) regulates cardiac contractility. We directly measure [Ca\(^{2+}\)]\(_{\text{SR}}\) in intact cardiac myocytes dynamically and quantitatively during beats, with high spatial resolution. Diastolic [Ca\(^{2+}\)]\(_{\text{SR}}\) (1 to 1.5 mmol/L) is only partially depleted (24% to 63%) during contraction. There is little temporal delay in the decline in [Ca\(^{2+}\)]\(_{\text{SR}}\) at release junctions and between junctions, indicating rapid internal diffusion. The incomplete local Ca\(^{2+}\) release shows that the inherently positive feedback of Ca\(^{2+}\)-induced Ca\(^{2+}\) release terminates, despite a large residual driving force. These findings place stringent novel constraints on how excitation-contraction coupling works in heart and also reveal a Ca\(^{2+}\) store reserve that could in principle be a therapeutic target to enhance cardiac function in heart failure. (Circ Res. 2003;93:663-670.)

**Key Words:** calcium homeostasis ■ sarcoplasmic reticulum ■ ryanodine receptors ■ confocal imaging ■ membrane transport

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Measurement of cytosolic free [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{c}\)) is routine in most cell types and central to understanding the critical and ubiquitous roles of [Ca\(^{2+}\)]\(_{c}\) in cellular signaling. In most cells, Ca\(^{2+}\) is stored in intracellular compartments, the endoplasmic or sarcoplasmic reticulum (ER or SR). The rapid release of this stored Ca\(^{2+}\) via inositol trisphosphate or ryanodine receptor (RyR) channels is the triggering event for many cellular signaling cascades, including muscle contraction. The total amount of Ca\(^{2+}\) stored in the SR ([Ca\(^{2+}\)]\(_{\text{SR}}\)) is critical to this Ca\(^{2+}\) signaling, by directly varying the amount available for release. In addition, in cardiac muscle, increasing [Ca\(^{2+}\)]\(_{\text{SR}}\) also increases fractional SR Ca\(^{2+}\) release for a given release trigger.1-4 This is probably due to an effect of luminal Ca\(^{2+}\) on RyR gating.5,6 The [Ca\(^{2+}\)]\(_{\text{SR}}\) dependence of release is nonlinear and extremely steep in the normal range of SR Ca\(^{2+}\) loads. Thus, variation in [Ca\(^{2+}\)]\(_{\text{SR}}\) may play an important role in regulating SR Ca\(^{2+}\) release.1-4

Although [Ca\(^{2+}\)]\(_{\text{SR}}\) is important, SR Ca\(^{2+}\) is heavily buffered, and it is free intra-SR [Ca\(^{2+}\)] ([Ca\(^{2+}\)]\(_{\text{SR}}\)) that centrally determines (1) [Ca\(^{2+}\)]\(_{\text{SR}}\), (2) the effect of intra-SR Ca\(^{2+}\) on the RyR, (3) the driving force for SR Ca\(^{2+}\) release, and (4) the maximal thermodynamic [Ca\(^{2+}\)] gradient that the SR Ca\(^{2+}\)-ATPase can establish. Knowledge about [Ca\(^{2+}\)]\(_{\text{SR}}\) is increasingly important in understanding cardiac excitation-contraction coupling (ECC) and numerous processes in virtually all cells. [Ca\(^{2+}\)]\(_{\text{SR}}\) can be measured by releasing SR Ca\(^{2+}\) by activation of RyR in intact cells (eg, by caffeine).7 This gives quantitative data about [Ca\(^{2+}\)]\(_{\text{SR}}\) but not [Ca\(^{2+}\)]\(_{\text{SR}}\). Intra-SR–trapped fluorescent Ca\(^{2+}\) indicators and Ca\(^{2+}\)-sensitive proteins targeted to organelles8-16 can assess [Ca\(^{2+}\)]\(_{\text{SR}}\), but truly quantitative data have been challenging to obtain, especially in cardiac muscle.

In the present study, we measure [Ca\(^{2+}\)]\(_{\text{SR}}\) directly in a spatially resolved, dynamic manner in intact ventricular myocytes. Because the data are spatial as well as quantitative, we also assess whether appreciable diffusional delays exist between [Ca\(^{2+}\)]\(_{\text{SR}}\) near release sites and sites far away.

Cardiac ECC works by local Ca\(^{2+}\)-induced SR Ca\(^{2+}\) release, where Ca\(^{2+}\) current is the trigger.3 This inherently positive feedback would be expected to empty the SR, although indirect evidence suggests that SR Ca\(^{2+}\) release is incomplete during a normal heartbeat.1-4 However, this is controversial and our understanding of cardiac ECC is limited by lack of knowledge of spatially resolved [Ca\(^{2+}\)]\(_{\text{SR}}\). Data presented here are critical to understanding how [Ca\(^{2+}\)]\(_{\text{SR}}\) is involved in regulating the release process.

**Materials and Methods**

**Myocyte Isolation and Indicator Loading**

Ventricular myocytes were isolated from New Zealand White rabbits as previously described2 and were loaded with Fluo-5N AM (Molecular Probes) for 2 hours, and then 1.5 hours was allowed for deesterification and outward leak of cytosolic indicator, all at 37°C.17 All experiments were performed at 23°C. Fluorescence was mea-

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sured both on confocal and epifluorescence microscopes at excitation=488 nm, emission >500 nm for Fluo-5N. Cells exposed to Di-8-ANNEPS to identify transverse tubules were imaged with excitation=488 nm and fluorescence emission at >600 nm. The image in Figure 2C was deconvolved as in Rios et al.18

All reagents and chemicals were purchased from Sigma Chemical Company except as indicated. Cell superfusate contained (in mmol/L) CaCl2 2, NaCl 140, KCl 4, MgCl2 1, HEPES 5, and glucose 10 (pH 7.4). Statistical significance was tested with two-way ANOVA. A value of P<0.05 was considered significant.

Fluo-5N Calibration
In vitro calibration was performed in intracellular solutions (control, in mmol/L, KCl 140, HEPES [pH 7.2]) with the [Ca2+] indicated. Solutions were suspended within a fluorometer with stirring, and Fluo-5N fluorescence was measured under the indicated conditions. In vivo, F0, was determined in an intact myocyte by adding 1 μmol/L isoproterenol (ISO), then subsequently adding 0.5 mmol/L tetracaine to block SR Ca2+ leak, and finally [Na+] was removed to raise both [Ca2+], and [Ca2+]SR. In vivo, ΔF/Δt was estimated in permeabilized cells (50 μg/mL saponin) with 10 mmol/L caffeine to allow [Ca2+] equilibration across the SR. Intracellular solution, as previously described,19 included (in mmol/L) Cs-glutamate 200, HEPES 10 (pH 7.2), Mg-ATP 5, phosphocreatine dithri 5, MgCl2 0.5, glutathione 10 (reduced form), 5 U/mL creatine phosphokinase, 8% dextran (MW 40 000), with variable free [Ca2+], 0.001 FCCP, 0.001 μmol/L ruthenium red, 0.002 μmol/L oligomycin, and 0.008 μmol/L cyclosporine to limit mitochondrial Ca2+ uptake.

Results
The rabbit ventricular myocyte shown in Figure 1A is loaded with Fluo-5N, a low-affinity Ca2+ indicator that has extremely low fluorescence when Ca2+-free.17 While there is surely some indicator in cytosol and mitochondria, [Ca2+] is submicromolar in these compartments, such that the fluorescence is primarily from the SR (where expected [Ca2+]SR is ~1 mmol/L). This SR localization is supported by 3 observations. First, the fluorescence pattern is localized to Z lines and transverse tubules (stained by the lipophilic fluorophore Di-8-ANNEPS), exactly as expected for cardiac junctional SR (JSR, Figures 2A and 2B). That is, there is higher fluorescence near transverse tubules (site of capacious JSR) and weaker fluorescence strands through the sarcomere (site of more wispy, less dense longitudinal or free SR). Moreover, [Ca2+]SR is expected to be the same throughout the resting SR, so brightness may reflect the expected ultrastructural SR organization. The periodic bright fluorescence regions (1.9-μm spacing; Figure 1B) correspond to sarcomeric spacing. Second, this distinct pattern is abolished by rapid application of 10 mmol/L caffeine, which causes SR Ca2+ release to the cytosol (Figure 1B). The remaining sporadic bright spots and perinuclear rings that are little affected by caffeine (and avoided in Figure 1B) probably reflect Fluo-5N compartmentalization in non-SR regions with high [Ca2+]. Third, permeabilization of the sarcolemma with saponin does not alter the fluorescence pattern appreciably (not shown). Thus, the image at high zoom in Figure 2C illustrates the anatomy of the SR as it wraps around the myofilaments (vertical dark regions within the sarcomere between wispy areas of FSR) with the junctional SR located at the Z lines.

Figure 1. Fluorescence signals from intra-SR Fluo-5N. A, Fluorescence is abolished by 10 mmol/L caffeine application at striations but not in non-SR regions. B, Enlargement of part of A (with intensity profile). Striations are periodic every 1.9 μm, consistent with intrasarcagnostic spacing and are abolished by caffeine.
[Ca^{2+}]_{SR} only partially recovers unless pacing is resumed. The caffeine-sensitive fluorescence (attributed to the SR) at 0.5-Hz stimulation is 53.2±1.8% (n=14, Figure 3Ab) of the total. All subsequent data refer only to this caffeine-sensitive component. During the twitch, fluorescence declines to a minimum of ~75% at ~100 ms after [Ca^{2+}]_{SR} starts to decline and recovers with a time constant (τ) of ~100 ms (Figure 3Ac). Figure 3B shows that the [Ca^{2+}]_{SR} depletion...
measured at a single SR junction in confocal microscopy is quite similar to the global [Ca$^{2+}$]_SR signal. We refer to these local [Ca$^{2+}$]_SR depletions as "Ca$^{2+}$ scraps" (since they are the intra-SR correlate of evoked Ca$^{2+}$ sparks). Moreover, when Ca$^{2+}$ scraps at junctional and free SR regions (as in Figure 2A) are compared, there is little kinetic difference (Figure 3D). This indicates that longitudinal diffusion within the SR is faster than we could readily detect (assuming SR Ca$^{2+}$ release occurs at JSR). Thus, Ca$^{2+}$ diffusion from free to junctional SR does not appreciably limit SR Ca$^{2+}$ availability for release, even at a single twitch. 

Figure 4A shows [Ca$^{2+}$]_SR and contractions at different pacing frequencies. As frequency increases, diastolic [Ca$^{2+}$]_SR increases, as does the extent of [Ca$^{2+}$]_SR depletion and the contraction amplitude. With $\beta$-adrenergic activation by ISO, there was a further increase in diastolic [Ca$^{2+}$]_SR and extent of [Ca$^{2+}$]_SR depletion and faster [Ca$^{2+}$]_SR recovery ($\tau$ decreased from 155 to 70 ms), consistent with the expected acceleration of SR Ca$^{2+}$ release occurs at JSR). Thus, Ca$^{2+}$ diffusion from free to junctional SR does not appreciably limit SR Ca$^{2+}$ availability for release, even at a single twitch.

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Because F may not be linearly related to [Ca$^{2+}$]_SR, calibrations are needed for greater quantitative evaluation. In vitro, Fluo-5N Ca$^{2+}$ affinity ($K_d=135$ $\text{mol/L}$) in solution measured in a fluorometer is not altered by Mg or tetracaine (used below), although tetracaine partially quenches fluorescence (Figure 5A). The presence of protein decreases both maximal fluorescence as well as apparent affinity of Fluo-5N for Ca$^{2+}$ (Figure 5A). At cellular protein concentrations (50 to 100 $\text{mg/mL}$), Fluo-5N affinity is reduced $\approx$3-fold (typical for fluorescent Ca$^{2+}$ indicators in cells). 

Maximal fluorescence (F$_{\text{Max}}$) in myocytes was defined by stimulating SR Ca$^{2+}$-ATPase by ISO and blocking SR Ca$^{2+}$ release by tetracaine (which dramatically increases SR Ca$^{2+}$ content). Note that F did not rise much on tetracaine addition (even when we accounted for the modest quench by tetracaine). To further raise [Ca$^{2+}$]_SR, extracellular Na$^+$ was abruptly removed (causing Ca$^{2+}$ entry via Na$^+$-Ca$^{2+}$ exchange), which caused spontaneous contractions and corre-
under these conditions (with the RyR inhibited), the SR \( \mathrm{Ca}^{2+} \) pump should approach a limiting \( [\mathrm{Ca}^{2+}]_{\text{SR}}/[[\mathrm{Ca}^{2+}]_{\text{i}} \) gradient, and \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) should rise by the same factor as \( [\mathrm{Ca}^{2+}]_{\text{i}} \) (and the elevation of average \( [\mathrm{Ca}^{2+}]_{\text{i}} \) is indicated by the cellular contracture in Figure 5B). Since \( F \) still did not increase appreciably, despite the substantial rise in \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) expected with this protocol, \( F_{\text{Max}} \) represents saturation of intra-SR Fluo-5N. \( F_{\text{Min}} \) is taken as \( F_{\text{Cal}} \).

To test whether \( K_{d}=400 \) \( \mu \text{mol/L} \) is appropriate for intra-SR Fluo-5N, we used permeabilized myocytes (with RyRs opened by caffeine). When \( [\mathrm{Ca}^{2+}]_{\text{i}} \) was stepped from 50 \( \mu \text{mol/L} \) \( (F_{\text{Min}}) \) to 400 \( \mu \text{mol/L} \) \( (F_{\text{400}}) \) to 10 mmol/L \( (F_{\text{Max}} \) Figure 3C), we found that \( F_{\text{400}} \) was, on average, 47.5\% of \( F_{\text{Max}} \). This confirms that 400 \( \mu \text{mol/L} \) is an appropriate \( K_{d} \) in situ.

Using these calibrations, we found that diastolic \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) increased from 0.48 to 0.92 to 1.65 mmol/L from 0.1 to 1 Hz (Figures 6A and 6B). These values are similar to time-averaged whole-heart NMR estimates of \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) \( (1.5 \) mmol/L) \( ^{12} \) and our estimates \( (\sim1 \) mmol/L), based on \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) and \( [\mathrm{Ca}^{2+}]_{\text{JSR}} \) in SR vesicles plus cellular \( [\mathrm{Ca}^{2+}]_{\text{SR}} \). However, both of those results lacked kinetic or spatial information.

The extent of \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) depletion increased from 24\% to 63\% over this range of frequencies. Diastolic ISO data were not calibrated because \( F_{\text{i}} \) was too near \( F_{\text{Max}} \). Minimum \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) attained during a twitch varied from 0.36 to 0.61 mmol/L (even with ISO). These \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) measurements support previous, less direct \( [\mathrm{Ca}^{2+}]_{\text{JSR}} \) measurements that suggested incomplete SR \( \mathrm{Ca}^{2+} \) depletion.\(^{1,2,4} \) Importantly, Figures 3B and 3C also indicate that JSR in individual junctions depletes only partially. These data are not consistent with recent models of ECC where local \( [\mathrm{Ca}^{2+}]_{\text{JSR}} \) was projected to be \( \sim95\% \) depleted early during the twitch, thereby limiting further SR \( \mathrm{Ca}^{2+} \) release.\(^{26} \)

**Discussion**

We report the first direct measurements of \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) during individual contractions and with subsarcomeric spatial resolution. These measurements and method provide valuable new quantitative information that is at the very heart of cardiac ECC. It has also been argued that SR \( \mathrm{Ca}^{2+} \) depletion does not participate in the shutoff of SR \( \mathrm{Ca}^{2+} \) release,\(^{21} \) and indeed we show that SR \( \mathrm{Ca}^{2+} \) release stops at local \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) \( \sim0.4 \) mmol/L when there is still a large driving force for SR \( \mathrm{Ca}^{2+} \) release.\(^{21} \) Cardiac ECC models have assumed that there is a major time delay (up to seconds) between recovery of \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) near Ca\( ^{2+} \) uptake sites (FSR) and at release sites (JSR).\(^{27} \) Such purported major time lags between JSR and FSR do not seem to occur during release (Figure 3C), and the \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) is restored rapidly during the twitch (even in the JSR). The apparent delay or restitution of SR \( \mathrm{Ca}^{2+} \) release (eg, at premature heartbeats) is probably due mainly to recovery of RyR (and/or L-type \( \mathrm{Ca}^{2+} \) channel) availability, rather than the amount of releasable SR \( \mathrm{Ca}^{2+} \).

We propose that \( [\mathrm{Ca}^{2+}]_{\text{SR}} \) depletion is dynamically involved in terminating SR \( \mathrm{Ca}^{2+} \) release, due to direct effects on
RyR gating (not by exhausting available SR Ca\(^{2+}\)). Indeed, the SR retains a Ca\(^{2+}\) reserve, which is pharmacologically accessible, as indicated by caffeine-induced Ca\(^{2+}\) transients, which completely deplete [Ca\(^{2+}\)]\(_{SR}\) (Figure 1B). When [Ca\(^{2+}\)]\(_{SR}\) is below 40% to 50% of its control value, resting SR Ca\(^{2+}\) leak (Ca\(^{2+}\) spark frequency) is very small and a normal Ca\(^{2+}\) current trigger fails to release appreciable SR Ca\(^{2+}\). This demonstrates that luminal [Ca\(^{2+}\)]\(_{SR}\) dynamically modulates SR Ca\(^{2+}\) release (and leak) during both diastole and ECC.

The fact that SR Ca\(^{2+}\) release does not go to completion even locally (as expected for positive feedback) rules out substrate limitation as the cause of release termination but leaves two potential types of inactivation. \(^{29,30}\) One mechanism, stochastic attrition, would be when a sufficient number of Ca\(^{2+}\) channels in a junction (L-type and RyR) close by chance to allow local [Ca\(^{2+}\)]\(_i\) to fall and break the positive feedback loop. This is unlikely to produce reliable termination of SR Ca\(^{2+}\) release, given the high number of channels at a junction (unless their gating is tightly coupled). \(^{21,29-31}\) The second major class would be a time-dependent RyR inactivation, which could depend explicitly on [Ca\(^{2+}\)]\(_i\), [Ca\(^{2+}\)]\(_{SR}\), or both. There is evidence for [Ca\(^{2+}\)]\(_i\)-dependent inactivation (or adaptation). \(^{32}\) DelPrincipe et al \(^{32}\) found that after a global cellular SR Ca\(^{2+}\) release, restitution required >1 second and suggested that SR Ca\(^{2+}\) depletion and slow functional repletion were the likely explanation (because discrete local Ca\(^{2+}\) releases showed much faster recovery). Our data indicate that the SR refills rather rapidly and suggests that this restitution depends more on recovery of RyR (or I\(_{Ca}\)) availability.

Interestingly, our own data demonstrate a trend upward in twitch minimum with increased release (Figure 4D). This would be consistent with a cytosolic Ca\(^{2+}\) -dependent inactivation site on the RyR, which binds more Ca\(^{2+}\), thus inactivating the channel faster and terminating release at a slightly higher [Ca\(^{2+}\)]\(_{SR}\). However, our observation that release stops (and can only be very weakly activated) when [Ca\(^{2+}\)]\(_{SR}\) <0.5 mmol/L also suggests an equally important regulatory role for [Ca\(^{2+}\)]\(_{SR}\) (perhaps by influencing how RyR responds to [Ca\(^{2+}\)]\(_i\)). \(^{5,6}\) Thus, both [Ca\(^{2+}\)]\(_{SR}\) and [Ca\(^{2+}\)]\(_i\), may both contribute dynamically to the shutoff of SR Ca\(^{2+}\) release. A corollary is that both RyR availability and [Ca\(^{2+}\)]\(_{SR}\) must recover to achieve restitution. So far, our results suggest that [Ca\(^{2+}\)]\(_{SR}\) recovers somewhat faster (\(\tau \approx 100\) ms) than does the RyR (\(\tau \approx 300\) ms).\(^{32,33}\) [Ca\(^{2+}\)]\(_{SR}\) dictates the effect of SR Ca\(^{2+}\) on RyR gating, the driving force for Ca\(^{2+}\) release, but it is also limited thermodynamically. For diastolic [Ca\(^{2+}\)]\(_i\)=100 to 150 mmol/L at 0.5 to 1 Hz (respectively), the [Ca\(^{2+}\)]\(_{SR}\)/[Ca\(^{2+}\)]\(_i\) gradient would be 9200 to 11 000. A gradient of 10 000 implies a high (78%) energetic efficiency for the SR Ca\(^{2+}\)-ATPase (for 2 Ca\(^{2+}\)/ATP) and a cytosolic \(\Delta G_{\text{ATP}}\) of 59 kJ/mol.\(^{25,34}\)

Our data provide the first direct quantitative examination of what is happening dynamically to local intra-SR free [Ca\(^{2+}\)] during cardiac ECC. This is especially important because [Ca\(^{2+}\)]\(_{SR}\) (rather than [Ca\(^{2+}\)]\(_i\)) is the central thermodynamic parameter that governs buffering, allosteric regulation, driving gradient, and transport limits. Thus, in cardiac myocytes, [Ca\(^{2+}\)]\(_{SR}\) is a major determinant of Ca\(^{2+}\) release and contraction. [Ca\(^{2+}\)]\(_{SR}\) may also limit cardiac function in heart failure.\(^{35,36}\) Intra-SR Ca\(^{2+}\) diffusion is rapid, and local [Ca\(^{2+}\)]\(_{SR}\) never gets much less than \(\approx 50\)% of its diastolic value, even with strong activation of ECC. The less than complete depletion of local [Ca\(^{2+}\)]\(_{SR}\) during normal SR Ca\(^{2+}\) release implies a residual SR Ca\(^{2+}\) reserve that might be pharmacologically accessible for treatment of diseases such as heart failure. The experimental approach described here should be very useful in further studies of SR Ca\(^{2+}\) in cardiac myocytes and other cell types. This novel approach should allow new mechanistic and quantitative questions to be addressed.

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**References**


**Figure 6.** Dynamic [Ca\(^{2+}\)]\(_{SR}\) profiles. A, Calibrated [Ca\(^{2+}\)]\(_{SR}\) signals. B, Mean diastolic [Ca\(^{2+}\)]\(_{SR}\) and fractional twitch depletion at different frequencies.


Ca\textsuperscript{2+} Scraps. Local Depletions of Free [Ca\textsuperscript{2+}] in Cardiac Sarcoplasmic Reticulum During Contractions Leave Substantial Ca\textsuperscript{2+} Reserve

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