Assessment of Sarcoplasmic Reticulum Ca^{2+} Depletion During Spontaneous Ca^{2+} Waves in Isolated Permeabilized Rabbit Ventricular Cardiomyocytes

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ABSTRACT In this study, Ca^{2+} release due to spontaneous Ca^{2+} waves was measured both from inside the sarcoplasmic reticulum (SR) and from the cytosol of rabbit cardiomyocytes. These measurements utilized Fluo5N-AM for intra-SR Ca^{2+} from intact cells and Fluo5F in the cytosol of permeabilized cells. Restricted subcellular volumes were resolved with the use of laser-scanning confocal microscopy. Local Ca^{2+} signals during spontaneous Ca^{2+} release were compared with those induced by rapid caffeine application. The free cytoplasmic [Ca^{2+}] increase during a Ca^{2+} wave was 98.1% ± 0.3% of that observed during caffeine application. Conversion to total Ca^{2+} release suggested that Ca^{2+} release from a Ca^{2+} wave was not significantly different from that released during caffeine application (104% ± 6%). In contrast, the maximum decrease in intra-SR Fluo-5N fluorescence during a Ca^{2+} wave was 82.5% ± 2.6% of that observed during caffeine application. Assuming a maximum free [Ca^{2+}] of 1.1 mM, this translates to a 96.2% ± 0.8% change in intra-SR free [Ca^{2+}] and a 91.7% ± 1.6% depletion of the total Ca^{2+}. This equates to a minimum intra-SR free Ca^{2+} of 46 ± 7 μM during a Ca^{2+} wave. Reduction of RyR2 Ca^{2+} sensitivity by tetracaine (50 μM) reduced the spontaneous Ca^{2+} release frequency while increasing the Ca^{2+} wave amplitude. This did not significantly change the total depletion of the SR (94.5% ± 1.1%). The calculated minimum [Ca^{2+}] during these Ca^{2+} waves (87 ± 19 μM) was significantly higher than control ($p < 0.05$). A computational model incorporating this level of Ca^{2+} depletion during a Ca^{2+} wave mimicked the transient and sustained effects of tetracaine on spontaneous Ca^{2+} release. In conclusion, spontaneous Ca^{2+} release results in substantial but not complete local Ca^{2+} depletion of the SR. Furthermore, measurements suggest that Ca^{2+} release terminates when luminal [Ca^{2+}] reaches ~50 μM.

INTRODUCTION

Under conditions of increased cellular Ca^{2+} load, ventricular cardiomyocytes exhibit spontaneous transient increases of cytoplasmic [Ca^{2+}] as a result of Ca^{2+} release from the sarcoplasmic reticulum (SR). The spontaneous increase of intracellular [Ca^{2+}] starts in a discrete area of the cardiomyocyte as a result of the opening of a cluster of SR Ca^{2+} release channels (type-2 ryanodine receptors (RyR2)). The release then propagates along the length of the myocyte as a Ca^{2+} wave. The mechanism for propagation is thought to involve a ''fire-diffuse-fire'' system (1), i.e., Ca^{2+} release from one cluster of RyR2s, followed by diffusion of cytosolic Ca^{2+} to a neighboring region of the SR, which causes firing of the adjacent RyR2 cluster via a process of Ca^{2+}-induced Ca^{2+} release. This mechanism contrasts with normal excitation-contraction (E-C) coupling, where Ca^{2+} is simultaneously released from the majority of RyR2 clusters throughout the cardiomyocyte after the synchronous influx of Ca^{2+} via L-type Ca^{2+} channels (2,3).

Although the mechanism of Ca^{2+}-induced activation of SR Ca^{2+} release is well characterized, the processes controlling the termination of the release process are less clear. Recent work suggests that reduction of the intra-SR [Ca^{2+}] acts to reduce the open probability of RyR2, thereby terminating release via regulatory proteins bound to the luminal surface of RyR2 (4–8). This is supported by recent work in which luminal Ca^{2+} was measured during Ca^{2+} release events (9–11). However, a number of issues remain uncertain. Primarily, it remains to be determined at what luminal [Ca^{2+}] the release process is terminated. Furthermore, luminal Ca^{2+} measurements in stimulated cardiomyocytes suggest a minimum free [Ca^{2+}] of ~0.5 mM (11). Estimates of depletion during spontaneous SR Ca^{2+} release in both intact and permeabilized cardiomyocytes range from 40% to 100% of the total SR content (12–15). This fraction is thought to change in the presence of agents that modulate the Ca^{2+} sensitivity of the RyR2, such as tetracaine or caffeine (15–17). In the study presented here, cytosolic and luminal Ca^{2+} measurements were used to determine the degree of Ca^{2+} depletion from the SR during a spontaneous Ca^{2+} wave.

MATERIALS AND METHODS

Cell isolation and permeabilization

New Zealand White rabbits (2–2.5 kg) were euthanized by administration of an intravenous injection of 500 IU heparin, together with an overdose of sodium pentobarbitone (100 mg · kg^{-1}). The hearts were removed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Ventricular myocytes were isolated from Langendorff-perfused rabbit hearts

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using a previously described enzymatic digestion method (18). Isolated cells were maintained in a modified Krebs solution (zero Ca^{2+}) at a concentration of ~10^4 cells/mL until use. The cells were allowed to settle onto the coverslip at the base of a cell perfusion bath. A mock intracellular solution (see below) containing β-escin (Sigma-Aldrich, Dorset, UK) was perfused at 0.1 mg · mL^{-1} for 0.5–1 min. When spontaneous oscillations were observed, the β-escin was removed.

Solutions

Permeabilized cells were perfused with a mock intracellular solution with the following composition (mM): 100 KCl, 5 K_2ATP, 5 Na_2PCr, 5.5 $MgCl_2$, 25 HEPES, 0.05 K_2EGTA, pH 7.0 (20–21°C). The [Ca^{2+}] in the perfusing solution was varied by the addition of known amounts of 1 M $CaCl_2$ stock solution (VWR, Leicestershire, UK). Free Ca^{2+} was confirmed using the intracellular calibration method (19) with solutions containing 10 mM EGTA. Free [Ca^{2+}] was calculated by means of a computer program (React; G.L. Smith, University of Glasgow, Glasgow, UK) with known affinity constants for H^+, Ca^{2+}, and Mg^{2+} for EGTA (20) and ATP and PCr (21). These chemicals were supplied by Sigma (Dorset, UK).

Measurements of cytoplasmic and SR [Ca^{2+}]

To monitor cytosolic [Ca^{2+}] within permeabilized cardiomyocytes, cells were perfused with a mock intracellular solution containing 10 μM Fluo-5F (Invitrogen). The methods associated with calibration and conversion of the Fluo-5F fluorescence signal to cytoplasmic [Ca^{2+}] were previously described in detail (14). Briefly, at the end of the experiment, cardiomyocytes were perfused with calibration solutions containing a range of [Ca^{2+}] buffered with 10 mM EGTA. The resulting intracellular fluorescence was used to calculate intracellular [Ca^{2+}] from the preceding fluorescence signal, assuming that the affinity of Ca^{2+} for Fluo-5F was 1.04 μM (22).

Intra-SR Ca^{2+} measurements were made by Sigma-Aldrich incubating cardiomyocytes with 10 μM Fluo-5N AM (Invitrogen, Paisley, UK) for 3–6 h at 37°C. Cytosolic dye was removed upon permeabilization, leaving the remaining dye trapped within intracellular organelles, including the SR. The SR-related component was assessed at the end of an experimental procedure by perfusing cardiomyocytes with 10 mM caffeine. Under these conditions, luminal [Ca^{2+}] was considered to be equilibrated across the SR membrane. Separate measurements established that the degree of chemical quenching of the Fluo-5N fluorescence by caffeine was minimal and had no significant consequences on the calculated values of intra-SR Ca^{2+}. Previous findings suggest that the maximum free [Ca^{2+}] achieved within the lumen of the SR is within the range of 0.7–1.5 mM (23). The recorded Fluo-5N fluorescence signals were converted to values of free luminal [Ca^{2+}] using the following assumptions: 1), the maximal fluorescence observed between Ca^{2+} waves corresponds to a free [Ca^{2+}] of 1.1 mM (taken as Fo); 2), the minimum fluorescence observed after perfusion with 10 mM caffeine is equal to the extracellular [Ca^{2+}]; and 3), the affinity of Fluo-5N for Ca^{2+} is 400 μM (11). The mean $\Delta F_{cs}/Fo$ was taken as the caffeine-sensitive change in fluorescence relative to Fo. Where total Ca^{2+} was calculated, it was expressed per liter cell cytosol, given an SR volume of 3.5% and a total cytosolic volume that was 65% of the cell (24). The K_d and B_{max} of intra-SR buffering were taken as 630 μM and 140 μmol/L cytosol, respectively (25). Cytosolic buffering was accounted for as previously described (14,25).

Confocal microscopy

Fluorescence confocal linescan images were recorded using a BioRad Radiance 2000. The Fluo-5F in the perfusing solution or the Fluo-5N in the SR was excited at 488 nm using a Kr/Ar laser and measured above 515 nm using the epifluorescence optics of a Nikon Eclipse inverted microscope with a Fluor 60× water objective lens (NA 1.2). The iris diameter was set at 1.9, providing an axial (z) resolution of ~0.9 μm and x-y resolution of ~0.5 μm based on full width at half-maximum amplitude measurements of images of 0.1 μm fluorescent beads (Invitrogen). Data were acquired in linescan mode at 2 ms/line, and the pixel dimension was 0.3 μm (512 pixels/scan; zoom = 1.4). The scanning laser line was oriented parallel with the long axis of the cell and placed approximately equidistant between the outer edge of the cell and the nucleus/nuclei to ensure that the nuclear area was not included in the scan line. To avoid movement artifacts, contraction was suppressed by inclusion of 10 μM cytochalasin D in the perfusing solution.

Image analysis

All image analysis was carried out using programs written in the Borland Delphi language, with the Pixelook library (http://plsoft.users.btopenworld.com/). Temporal offsetting of Ca^{2+} wave linescan images was only carried out on wavefronts with homogeneous velocity over at least 40 μm. To acquire accurate measurements of the time course and amplitude of the fluorescence changes that occurred during the Ca^{2+} waves, >100 pixelwide sections (~1/3 of the cell) with homogeneous velocity were averaged. Because of the nonsynchronous nature of the propagated release, velocity was corrected for by offsetting each pixel by the amount required to produce a new image with the linescan realigned to the same time for the individual spontaneous release event (Figs. 1 *A* and 2 *A*). This was accomplished by means of the autocorrelation method used by Cheng et al. (26). This realignment allowed accurate resolution of the amplitude and time course of the Ca^{2+} wave, minimizing temporal smearing of the mean fluorescence signal from the selected region. This technique was applied to signals derived from the cytoplasmic Ca^{2+} indicator (Fluo-5F; see Fig. 1 *A*) and intra-SR Ca^{2+} indicator (Fluo-5N; see Fig. 2 *A*).

Computational modeling

A previously published (14) three-compartment model consisting of 1), the SR; 2), the cytosol; and 3), the extracellular compartments of the cell allowed reconstruction of the changes in Ca^{2+} observed (see *inset* in Fig. 5 for diagram of fluxes). Estimates of the magnitude and time course of the various Ca^{2+} flux pathways were derived from experimental measurements in permeabilized cardiac myocytes. Cytoplasmic [Ca^{2+}] from a resolution-limited volume of a cardiac myocyte during the Ca^{2+} wave was calculated from confocal Fluo-5F fluorescence signals. These values, in conjunction with estimates for intracellular buffering and the diffusion rate constant, were used to calculate the underlying Ca^{2+} fluxes. This approach was based on the assumption that once initiated, the Ca^{2+} wave in cardiac muscle is a one-dimensional wave, i.e., effectively, the Ca^{2+} wave occurs simultaneously throughout the depth and width of the cell. SR Ca^{2+} release was initiated when luminal [Ca^{2+}] reached a threshold of 1.1 mM. The magnitude and time course of the change in RyR2 permeability during a Ca^{2+} wave was based on experimental measurements (14). The application of tetracaine was modeled by increasing the luminal [Ca^{2+}] necessary to trigger release by 40%. This increase approximated experimentally observed values (see Fig. 3).

Statistics

Data are shown as means ± standard error (SE) and were compared with the use of Students' t-test in all cases, with significance accepted at $p < 0.05$.

RESULTS

Comparison of cytoplasmic Ca^{2+} signals during a Ca^{2+} wave and caffeine-induced Ca^{2+} release

During a spontaneous wave, cellular Ca^{2+} rises to ~1.5 μM as a result of release from the SR. This then falls, mostly due to uptake via sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA), but also as a result of a loss via diffusion across the permeabilized membrane. It was previously shown that

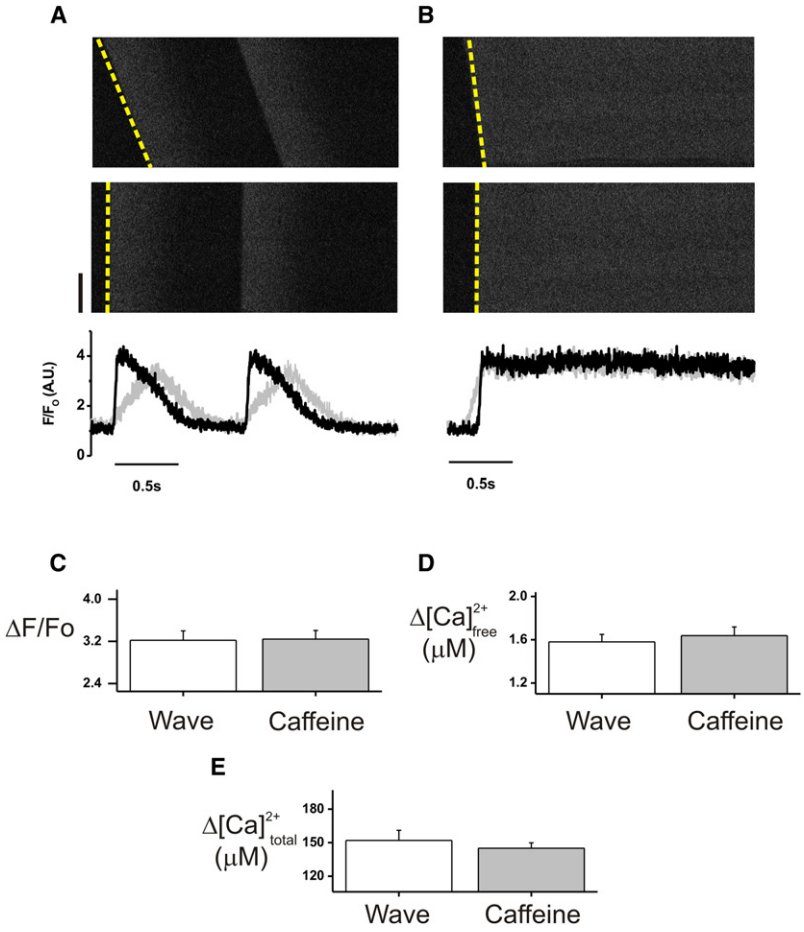

FIGURE 1 Measurement of cytosolic Ca^{2+} during spontaneous Ca^{2+} waves and caffeine-induced Ca^{2+} release. (*A*) Upper: Typical confocal linescan image of two Ca^{2+} waves from a single permeabilized cardiomyocyte perfused with 600 nM Ca^{2+} (in 50 μM EGTA, 10 μM Fluo-5F). The image is taken from approximately half the length of the cell (*black bar* indicates 20 μm). Middle: Image from the upper panel after temporal realignment to allow for analysis of the propagation velocity (~160 μms^{-1}). Lower: Normalized fluorescence traces corresponding to the original (*black*) and aligned (*gray*) images. (*B*) The same cell during a caffeine-induced Ca^{2+} release, with the original (*upper*) and aligned (*middle*) images and their corresponding fluorescence traces (*lower*). Bar graphs show a comparison of the amplitude of spontaneous Ca^{2+} wave versus caffeine in terms of F/Fo (*C*), free cytosolic [Ca^{2+}] (*D*), and total cytosolic [Ca^{2+}] (*E*).

the diffusional loss is recovered as SERCA lowers the cellular Ca^{2+} below that of the bulk extracellular perfusate (to reach a minimum of ~150 nM), and that over a period of three to five waves, the mean extra- and intracellular [Ca^{2+}] levels are balanced. Fig. 1 *A* shows a linescan image from a permeabilized rabbit cardiomyocyte. The perfusing solution contained ~600 nM Ca^{2+}; under these conditions, Ca^{2+} waves occurred spontaneously at a rate of ~0.4 Hz. The Ca^{2+} waves shown propagate along the length of the cell at a constant velocity (173.3 $\pm$ 9.7 μms^{-1}, $n = 7$). The slow rate of rise of the mean fluorescence signal is due to the smearing of a more rapid upstroke by the nonsynchronous nature of the Ca^{2+} wave. This is evident from Fig. 1 *A* (*middle*), where the linescan image was corrected for a constant propagation velocity. The mean fluorescence from the corrected fluorescence signal shows the rapid upstroke associated with the Ca^{2+} wave. Fig. 1 *B* (*upper*) shows the linescan image from the same permeabilized cardiomyocyte upon rapid application of 10 mM caffeine. The rise of fluorescence is almost synchronous along the length of the cell (velocity ~800 μms^{-1}). The linescan image was corrected for the small delay in Ca^{2+} release that occurred along the length of the cell (Fig. 1 *B*, *middle*). After correction, the amplitude of the fluorescence transient was comparable to that of the corrected Ca^{2+} wave signals, indicating that equivalent amounts of Ca^{2+} were released from the SR during both events. These signals can be converted to free [Ca^{2+}] changes with the use of calibration solutions applied at the end of the protocol (14). The free cytoplasmic [Ca^{2+}] was subsequently converted to total cytoplasmic [Ca^{2+}] using published values of the cytoplasmic buffering (27). The mean values for Ca^{2+} wave amplitude and the subsequent caffeine-induced Ca^{2+} release are shown in Fig. 1 *C*. On average, the amplitude of change in cytoplasmic [Ca^{2+}] during a Ca^{2+} wave was 98.1% $\pm$ 0.3% ($n = 7$) of that induced by caffeine. When the total cytoplasmic Ca^{2+} signals were compared, the increase in total [Ca^{2+}] during a Ca^{2+} wave was 104% $\pm$ 6% ($n = 7$) of that from a caffeine-induced release. The larger error in the latter measurement is due to the variation in initial values of free [Ca^{2+}] used to calculate the change in total cytoplasmic [Ca^{2+}]. These data indicate that the total Ca^{2+} released from the SR during a Ca^{2+} wave was indistinguishable from that released by rapid application of caffeine, and suggests complete depletion of the SR during a Ca^{2+} wave. The rise in total cytoplasmic [Ca^{2+}] as a result of rapid caffeine application was 164 $\pm$ 6 μM (Fig. 1 *E*), which translates to a total [Ca^{2+}] within the SR before release of 2.83 $\pm$ 0.11 mM and a free [Ca^{2+}] of 1.12 mM using previously published values of SR volume and buffering (25).

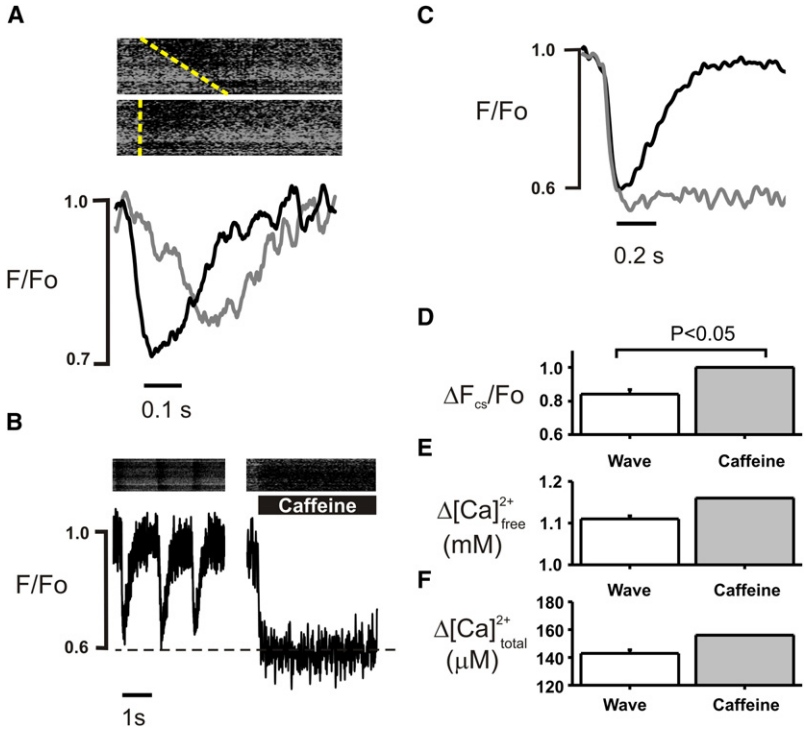

FIGURE 2 Measurement of luminal Ca^{2+} in spontaneous Ca^{2+} waves and caffeine-induced Ca^{2+} release. (*A*) Confocal linescan image of cardiomyocyte loaded with fluo-5N-AM before (*upper*) and after (*middle*) correction for propagation. Lower: Resultant fluorescence trace from the original (*gray line*) and corrected (*black line*) images. Fluorescence was normalized to F/Fo, where Fo is the fluorescence recorded just before release occurred. (*B*) Temporally realigned fluorescence signal indicating changes in SR content during spontaneous and caffeine-induced release. (*C*) Superimposed signals from caffeine (*gray*) and spontaneous release (*black*). Bar graphs of F_{cs}/Fo amplitude (*D*), free [Ca^{2+}]SR (*E*), and total [Ca^{2+}]SR (*F*).

Comparison of luminal Ca^{2+} signals during a Ca^{2+} wave and caffeine-induced Ca^{2+} release

Fig. 2 shows Ca^{2+} waves and caffeine-induced release examined from the perspective of intra-SR Ca^{2+} by loading the lumen of the SR with the low-affinity dye Fluo-5N. After permeabilization and perfusion with a mock intracellular solution, the staining of the cardiac cell showed a marked striated pattern comparable to that observed in previous studies (10,28). Perfusion with a mock intracellular solution containing 600 nM Ca^{2+} caused spontaneous Ca^{2+} release to occur at a frequency of ~0.4 Hz. Linescan images indicated transient decreases in fluorescence that propagated along the length of the cell with a velocity similar to that observed with signals derived from the cytoplasmic indicator Fluo-5F (172 $\pm$ 6 μms^{-1}, $n = 9$). Rapid application of caffeine caused a rapid decrease in fluorescence uniformly along the length of the cardiomyocyte. The fluorescence signals derived from both spontaneous Ca^{2+} waves and rapid caffeine application were corrected for their associated propagation velocity by using the same realignment process applied to the cytoplasmic signals (see Fig. 2 *A*). Fig. 2 *B* shows three sequential waves of approximately equal velocity realigned to show the relative fluorescence change compared to the caffeine response in the same cell. These waves show an ~80% reduction in relative fluorescence compared to the caffeine response. The mean cellular fluorescence derived from the corrected signal in Fig. 2 *B* is shown in Fig. 2 *C*. The rapid decrease in fluorescence associated with a Ca^{2+} wave was routinely smaller in amplitude than that observed during rapid caffeine application, with a mean decrease in the fluorescence signal associated with a Ca^{2+} wave 82.5% $\pm$ 2.6% ($n = 7$) of that observed on rapid application of caffeine.

Using the previously published value of the affinity constant for Ca-Fluo5N (29), and assuming a maximum intra-SR [Ca^{2+}] before release of 1.1 mM, the decrease in free intra-SR [Ca^{2+}] during a Ca^{2+} wave was 96.2% $\pm$ 0.7% ($n = 7$) of that observed upon rapid addition of caffeine. The calculations suggest that during a Ca^{2+} wave, the minimum intra-SR free Ca^{2+} was 46 $\pm$ 7 μM ($n = 7$). These values were converted to changes in total Ca^{2+} within the SR using previously published values of the intra-SR Ca^{2+} buffering power (27). The results predicted that the total SR [Ca^{2+}] would decrease by 2.46 $\pm$ 0.04 mM and the total cytoplasmic Ca^{2+} would increase by 143 $\pm$ 2.5 μM ($n = 7$) (Fig. 2 *F*). The measurements indicate that the total SR [Ca^{2+}] during a Ca^{2+} wave decreased by 91.7% $\pm$ 1.6% of that observed during rapid caffeine administration. This value is significantly different from 100% ($p < 0.05$) and indicates that despite the significant amount of Ca^{2+} remaining within the SR during a Ca^{2+} wave (46 μM free, 142 μM total), the total Ca^{2+} released was ~92% of that within the SR. The mean values of fluorescence change, change in free Ca^{2+}, and total Ca^{2+} released during both a Ca^{2+} wave and caffeine application are given in Fig. 2, *D–F*. Note that the increase in total cellular [Ca^{2+}] for a caffeine-induced release is ~160 μM, and this value is within 5% of the value calculated from cytosolic signals (Fig. 1 *E*). These observations predict that SR depletion is close to complete during a Ca^{2+} wave.

Effects of tetracaine on spontaneous SR Ca^{2+} release and caffeine-induced Ca^{2+} release

The effects of tetracaine (50 μM) on spontaneous Ca^{2+} release are shown in Fig. 3. Previous studies of permeabilized (17) and intact rat cardiomyocytes (16,30) showed that addition of tetracaine transiently suppressed the spontaneous Ca^{2+} release. When SR Ca^{2+} release returned, the frequency of spontaneous release was lower than control values, whereas the amplitude of the Ca^{2+} wave was increased. Removal of tetracaine caused a transient burst of higher-frequency spontaneous Ca^{2+} waves before amplitude and frequency returned to control values. The changes in the spontaneous Ca^{2+} wave frequency are shown in detail in Fig. 3 *A* (*upper*). Fig. 3 *B* shows typical Ca^{2+} signals derived from velocity-corrected linescan signals before and during application of tetracaine. The larger transient amplitudes recorded in tetracaine were of comparable amplitude to the Ca^{2+} release caused by rapid caffeine application. The mean values of the Ca^{2+} signals from a number of experiments are shown in Fig. 3, *C–E*. These data show that addition of tetracaine caused a significant increase in Ca^{2+} wave amplitude and reduced Ca^{2+} wave frequency (Fig. 3, *C* and *D*). Quantification of the Ca^{2+} released from the SR during Ca^{2+} waves indicated that, as seen in the absence of tetracaine, the SR Ca^{2+} release could not be distinguished from caffeine-induced release (97.3% $\pm$ 0.4%). The increased amplitude of both release events indicated an increased SR Ca^{2+} content in tetracaine of ~30%. Using previously published values of intra-SR buffering, this would predict a maximum free [Ca^{2+}] within the SR of 1.58 $\pm$ 0.08 mM

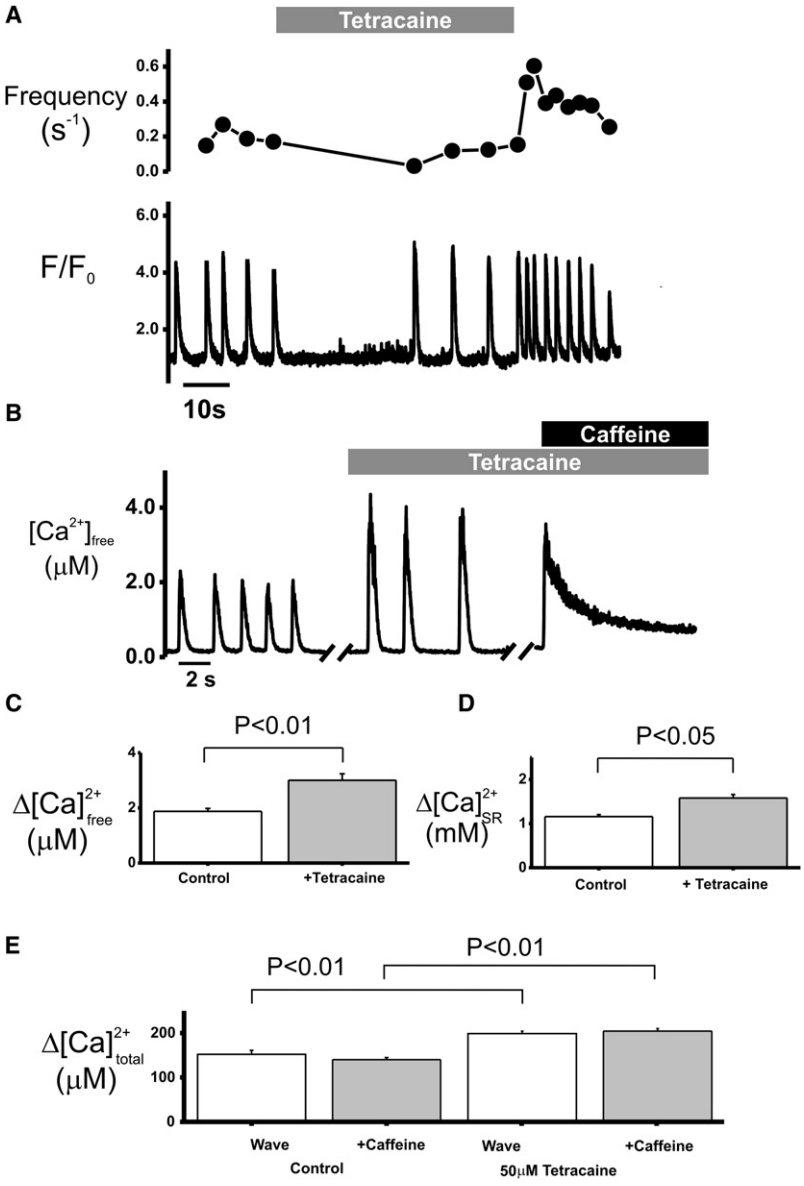

FIGURE 3 Effect of 50 μM tetracaine on spontaneous Ca^{2+} release in permeabilized cardiomyocytes. (*A*) Traces show spontaneous Ca^{2+} wave frequency (*upper*) and corresponding F/Fo (*lower*), showing the quiescent period, steady state, and rapid burst of releases on removal of tetracaine. (*B*) Traces from linescan recordings from a different cell after conversion to free Ca^{2+} from a typical cell in control conditions (600 nM Ca^{2+}, Fluo-5F). After steady state is achieved, larger, less frequent spontaneous releases occur. The amplitude of caffeine-induced release in the presence of tetracaine is similar to that of spontaneous release. Waves were corrected for temporal smearing. Bar graphs showing mean data $\pm$ SE comparing values for free [Ca^{2+}] (*C*), SR [Ca^{2+}] (*D*), and total [Ca^{2+}] (*E*), without (control) and in the presence of 50 μM tetracaine ($n = 6$).

in tetracaine before release, a value significantly higher than that calculated under control conditions ($p < 0.01$).

Comparison of luminal Ca^{2+} signals during a Ca^{2+} wave and caffeine-induced Ca^{2+} release after tetracaine addition

Fig. 4 shows the effect of tetracaine on spontaneous Ca^{2+} release using intra-SR signals derived from luminal Fluo-5N. On application of tetracaine, there was a modest elevation of the fluorescence signal before release, indicating an increased luminal [Ca^{2+}]. An increased luminal [Ca^{2+}] was observed in ~25% of the cells. The lack of a consistent increase despite a ~30% increase in SR Ca^{2+} release observed in cytoplasmic signals can be explained by the poor sensitivity of the luminal indicator in this range of Ca^{2+} (29). Although the dye has a relatively low Ca^{2+} affinity, the conversion of Fluo-5N fluorescence to SR [Ca^{2+}] will be associated with a 2.5-fold higher error at 1.6 mM compared to 1.1 mM (see Fig S1 in the Supporting Material). The decrease in Ca^{2+} wave frequency when tetracaine was applied indicated an effect comparable to those observed using the cytoplasmic indicator. Fig. 4 *A* (*upper*) shows fluorescence signals during a Ca^{2+} wave and caffeine application in the absence and presence of tetracaine. Fig. 4 *A* (*lower*) shows a typical signal from spontaneous and caffeine-induced Ca^{2+} release in the presence of tetracaine. Fig. 4, *B–D*, show the mean values of SR depletion during a Ca^{2+} wave and caffeine application. Assuming a maximum free [Ca^{2+}] of 1.6 mM, a Ca^{2+} wave in tetracaine caused a change in free [Ca^{2+}] of 94.5% ± 1.1% ($n = 6$), a change in total [Ca^{2+}] of 88.1% ± 2.2% and a minimum [Ca^{2+}] of 87 ± 19 μM. These data suggest that inhibition of RyR2 by tetracaine does not dramatically modify the fractional release of Ca^{2+} from the SR; the larger amount of Ca^{2+} release is achieved by an increased threshold [Ca^{2+}] for spontaneous release.

Computational modeling of the response of SR to tetracaine addition

In previous work, we established a computational model of spontaneous Ca^{2+} release with values of diffusion coefficient, SR Ca^{2+} uptake, and release fluxes that were based on measurements from permeabilized rabbit cardiomyocytes (14). This model was used to test whether completely depleting the SR was capable of reproducing the behavior

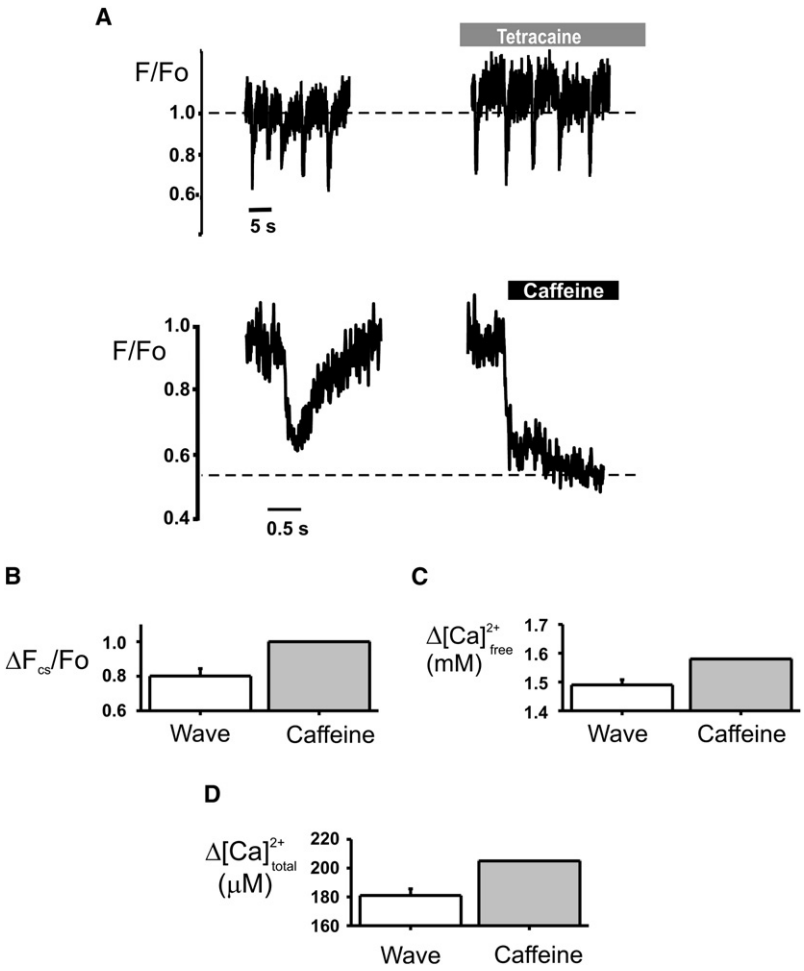

FIGURE 4 Effect of 50 μM tetracaine on luminal Ca^{2+} in spontaneous Ca^{2+} waves. (*A*) Upper: Fluo-5N fluorescence signal before and after application of 50 μM tetracaine, demonstrating a decrease in spontaneous Ca^{2+} release frequency. Lower: An averaged Ca^{2+} wave signal from the last four transients and the subsequent caffeine application (20 mM) (*black bar*). Bar graphs show mean $\Delta F_{cs}/Fo$ (*B*), ΔCa^{2+} free (*C*), and ΔCa^{2+} total (*D*) ($n = 6$) during wave- and caffeine-induced releases. No significant differences were found.

of intact and permeabilized cardiac myocytes. As demonstrated above, lowering the Ca^{2+}-sensitivity of RyR2 using tetracaine increased the apparent SR Ca^{2+} threshold for spontaneous Ca^{2+} release. These changes were imposed on a computational model of SR Ca^{2+} release as shown in Fig. 5 *A*. The threshold for release was raised from an SR free [Ca^{2+}] of 1.16 mM to 1.58 mM. This resulted in an inhibition of spontaneous release as the Ca^{2+} content of the SR was filled to the new higher threshold value. Spontaneous Ca^{2+} release was then reestablished with a new steady state at a lower rate of oscillation. Restoration of the control threshold for SR Ca^{2+} release (i.e., removal of tetracaine) induced a burst of high-frequency spontaneous Ca^{2+} releases very similar to those observed experimentally (Fig. 3 *A*). Fig. 5 *B* shows a detailed time course of the individual Ca^{2+} release events from selected parts of the trace. In addition to free cytoplasmic [Ca^{2+}] and free luminal [Ca^{2+}], the calculated rate of SERCA-mediated Ca^{2+} uptake is also shown. The higher rate of spontaneous release on removal of tetracaine can be explained by the extended phase of maximal SR Ca^{2+} uptake after release. This is retained for several seconds after the normal threshold for SR Ca^{2+} release is restored. The higher rate of SR Ca^{2+} uptake is the result of a higher cytoplasmic [Ca^{2+}] during the decay phase of the spontaneous Ca^{2+} release. This phase is prolonged because the minimum diastolic [Ca^{2+}] remains increased for several seconds after the removal of tetracaine. During this phase, total cellular Ca^{2+} will gradually return to control levels (Fig. 5 *A*, after *red circle*), with a net loss of Ca^{2+} due to diffusion across the permeabilized surface membrane.

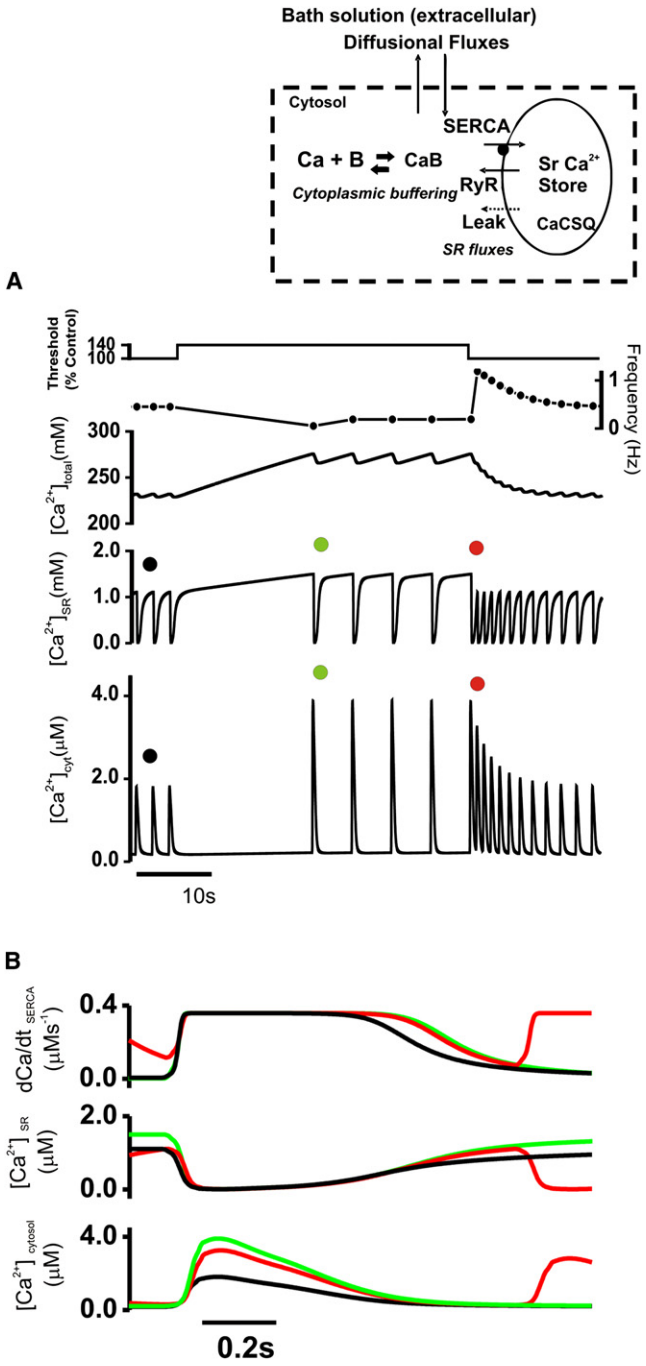

FIGURE 5 Data from a three-compartment simulation of spontaneous Ca^{2+} release, showing the effects of an increased SR release threshold. (*Inset*) Three-compartment model diagram outlining the Ca^{2+} fluxes and buffering involved in the extracellular, cytosolic, and SR compartments. (*A*) Traces showing the threshold for release, Ca^{2+} wave frequency, total cell [Ca^{2+}], SR [Ca^{2+}], and cytosolic Ca^{2+}. Circles denote the starting points of traces of corresponding colors in *B*. (*B*) Rescaled trace of data in *A*.

DISCUSSION

Spontaneous Ca^{2+} release in cardiac cells is thought to be caused by an elevated SR [Ca^{2+}] that sensitizes clusters of RyR2s to cytosolic Ca^{2+}, resulting in channel opening. Similarly, termination of release is thought to occur when SR Ca^{2+} falls to a level that desensitizes the cytosolic Ca^{2+} release site to close the RyR2. Currently, there is no consensus about the level of SR depletion required to terminate spontaneous Ca^{2+} release.

In this study, SR Ca^{2+} release during spontaneous Ca^{2+} waves in permeabilized ventricular myocytes was used as a tool to elucidate the mechanisms underlying RyR2 function. Cytoplasmic Ca^{2+} measurements indicated that the amount of Ca^{2+} released from the SR during a Ca^{2+} wave was indistinguishable from that produced by rapid caffeine application. Therefore, these measurements cannot resolve whether any intra-SR Ca^{2+} remains after Ca^{2+} release. In contrast, measurements of intra-SR free [Ca^{2+}] indicated that depletion of the SR during a Ca^{2+} wave was significantly less than that observed during caffeine application. This apparent contradiction was resolved when the intra-SR [Ca^{2+}] signals were converted to changes in total [Ca^{2+}]; under these circumstances, the Ca^{2+} release during a Ca^{2+} wave resulted in a degree of SR depletion that was ~96% of the total available Ca^{2+}, a value well within the error of the measurements from cytoplasmic [Ca^{2+}] signals.

Assessment of SR depletion using cytoplasmic Ca^{2+} sensitive indicators

This study shows that when the cytoplasmic Ca^{2+} indicator Fluo-5F is used, the amplitude of the Ca^{2+} transients caused by a spontaneous Ca^{2+} wave is only ~3% smaller than that caused by rapid caffeine application. This result contrasts with previous work on whole cell Ca^{2+} and shortening, which indicated that spontaneous Ca^{2+} release caused a much smaller rise of intracellular Ca^{2+} or cell shortening compared to caffeine-induced release (15). The explanation lies in the temporal smearing of the propagated Ca^{2+} wave. Normally, a Ca^{2+} wave takes ~1 s to propagate along the length of the cell at room temperature. As demonstrated in Fig. 1, at any one time over this period only ~30% of the volume of the cardiomyocyte experiences an increased cytoplasmic $[Ca^{2+}]$, whereas ~70% of the volume is either awaiting the arrival of the Ca^{2+} wave or is recovering after the release phase. In contrast, caffeine releases Ca^{2+} synchronously throughout the cardiomyocytes; therefore, local and whole cell signals are comparable.

Allowing for differences in initial cytoplasmic $[Ca^{2+}]$ values while calculating the increase in total $[Ca^{2+}]$ during a Ca^{2+} wave and from rapid caffeine application did not reveal significantly different values between these two Ca^{2+} release events, as the variability prevented differences of $<10\%$ from being distinguished. The higher variability arises from the uncertainties over the resting (minimum) free $[Ca^{2+}]$ and peak (maximum) free $[Ca^{2+}]$, which translates to much larger variations in total cellular $[Ca^{2+}]$. The use of an alternative Ca^{2+}-sensitive dye would not reduce these errors, because although an indicator with a lower Ca^{2+} affinity than Fluo-5N may provide better resolution of the peak Ca^{2+} signal, estimation of the minimum Ca^{2+} values would be less precise.

Assessment of SR depletion using luminal Ca^{2+} indicators

The staining pattern of Fluo-5N AM-loaded cardiomyocytes observed in this study is similar to that published in previous studies (10,28) and consistent with the majority of the dye accumulating in the terminal cisternae of the SR. When velocity-correction techniques were applied to the linescan images of Fluo-5N fluorescence signals, the amplitude of the fluorescence change associated with a Ca^{2+} wave was only 82% of that observed during a rapid caffeine application. This value was similar to the approximate value reported in an earlier study (10). Assuming that caffeine allows equilibration of the $[Ca^{2+}]$ between intra-SR and cytoplasmic compartments, the fluorescence signal at the peak of the Ca^{2+} wave translates to a minimum intra-SR $[Ca^{2+}]$ of ~47 μM. With the additional assumption that the maximum free intra-SR $[Ca^{2+}]$ signal between Ca^{2+} waves is 1.1 mM, the change in free $[Ca^{2+}]$ during a Ca^{2+} wave is ~96% of that during caffeine exposure. Converting these values to total Ca^{2+} values suggests that the total Ca^{2+} released from the SR during a wave is ~92% of that released during caffeine. These values are comparable to the estimates provided by the cytoplasmic Ca^{2+} signals and suggest that although SR Ca^{2+} release terminates before complete SR depletion, the total Ca^{2+} released is >90% of the total SR content. As shown in Fig. 6, this estimate of the degree of depletion was relatively insensitive to the assumed maximal free $[Ca^{2+}]$ within the SR, indicating that this was not a crucial parameter in this calculation. These measurements were made at room temperature (20°C) and should be repeated at 37°C. An increased temperature will alter the K_d and V_{max} of SERCA, and may change the affinity of cytoplasmic and intra-SR buffers. None of these changes will alter the fractional SR Ca^{2+}-release. However, the temperature dependence of RyR2 in terms of Ca^{2+}

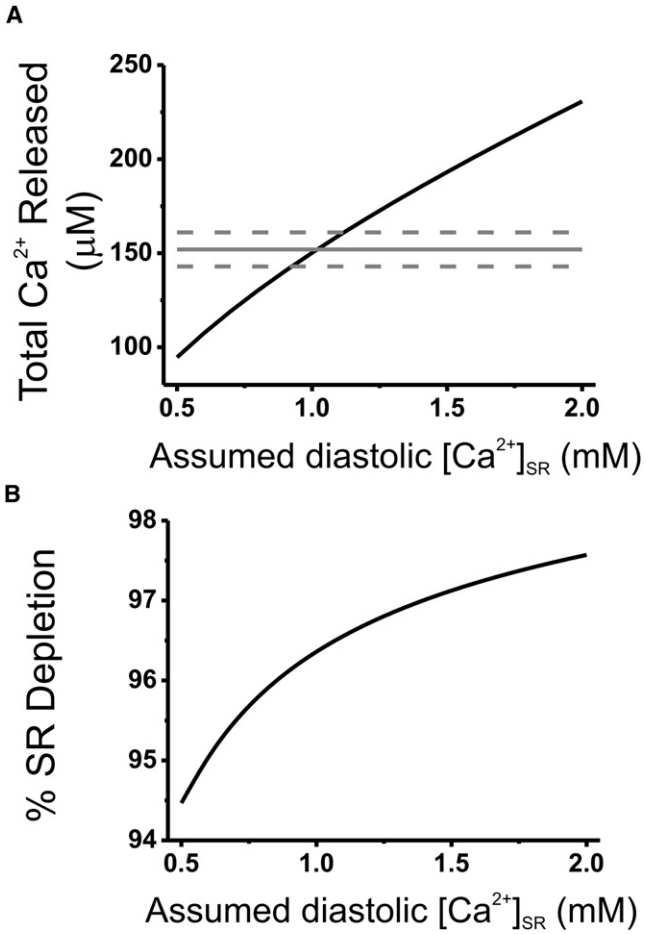

FIGURE 6 Calculated relationship between assumed maximal diastolic $[Ca^{2+}]$ and % SR depletion based on a range of "assumed" resting SR $[Ca^{2+}]$ values. (*A*) The solid black line is the relationship between assumed diastolic Ca^{2+} and the calculated total Ca^{2+} released during a Ca^{2+} wave. Gray lines indicate measured values of total Ca^{2+} released from the SR during a Ca^{2+} wave (based on cytosolic Ca^{2+} measurements). Dotted lines indicate ±10%. (*B*) Calculated % depletion of the SR during a Ca^{2+} wave, assuming a minimum $[Ca^{2+}]$SR of 50 μM and a range of values of maximal diastolic $[Ca^{2+}]$SR.

sensitivity and cooperative function within the cluster is unclear; both of these factors will determine the degree of depletion at 37°C.

Future studies will also determine whether the level of intra-SR Ca^{2+} remaining after a Ca^{2+} wave is similar when Ca^{2+} release is activated via E-C coupling mechanisms. Previous measurements of SR depletion on whole intact cardiomyocytes indicated a minimum SR Ca^{2+} of 0.5 mM across a range of inotropic levels (11). This is considerably higher than the ~50 μM reported in this study on permeabilized cells, and implies a large reserve remaining within the SR. Further studies are required to investigate the reasons for this discrepancy, which may lie in the efficiency of E-C coupling and/or in the difference between local and global depletion measurements.

Implications for the control of Ca^{2+} release from the SR

The data presented in this study can be used to describe a simple model for the control of Ca^{2+} release from the SR by both cytoplasmic and intra-SR control processes. As illustrated in Fig. 7, before Ca^{2+} release, cytoplasmic [Ca^{2+}] adjacent to the cluster of RyR2 is ~100 nM and the intra-SR [Ca^{2+}] approaches 1.1 mM due to SERCA-mediated Ca^{2+} uptake. As intra-SR [Ca^{2+}] increases, the open probability (Po) of RyR2 increases due to the action of Ca^{2+}on luminal regulatory proteins (junctin/triadin/calsequestrin). When intra-SR free [Ca^{2+}] approaches 1 mM, the Po of RyR2 increases dramatically and triggers Ca^{2+} efflux from the RyR2 cluster, thereby depleting intra-SR [Ca^{2+}] and increasing the [Ca^{2+}] on the cytoplasmic surface of the RyR2 cluster. The raised cytoplasmic [Ca^{2+}] will further activate the RyR2 cluster via the Ca^{2+}-induced Ca^{2+}-release process. The sustained increase in cytoplasmic [Ca^{2+}] would maintain a high RyR2 permeability, effectively shifting the intra-SR Ca^{2+} dependence of RyR2 activity to the left (see Fig. 7). Under these circumstances, intra-SR Ca^{2+} would continue to decrease during the release phase until levels reach ~50 μM. At this point, intra-SR regulatory processes would reduce the RyR2 Po sufficiently to terminate release, thereby allowing the cytoplasmic Ca^{2+} to start to decrease. This process would occur on a timescale of <50 ms.

In theory, the mechanism proposed in Fig. 7 could be employed to explain the graded nature of fractional SR Ca^{2+} release observed during physiological E-C coupling (11). The graded release can be explained in terms of the interplay of the Ca^{2+}-sensing sites on the luminal and cytoplasmic faces of the RyR2 cluster. To explore one example, the relative depletion of SR Ca^{2+} during E-C coupling is lower when the SR Ca^{2+} content is low (11). This result can be explained in terms of a reduced number of release clusters activated and partial depletion of the active release sites. Low luminal [Ca^{2+}] will reduce the Ca^{2+} sensitivity of the cytoplasmic sensors to Ca^{2+}. This will reduce the probability of the cluster firing, thereby reducing the number of active clusters recruited by the L-type Ca^{2+} current trigger. The active clusters will then experience a reduced dyadic [Ca^{2+}] due to the lower [Ca^{2+}] gradient across the SR membrane. This will limit the activation of RyR2 and therefore encourage the premature termination of the release event.

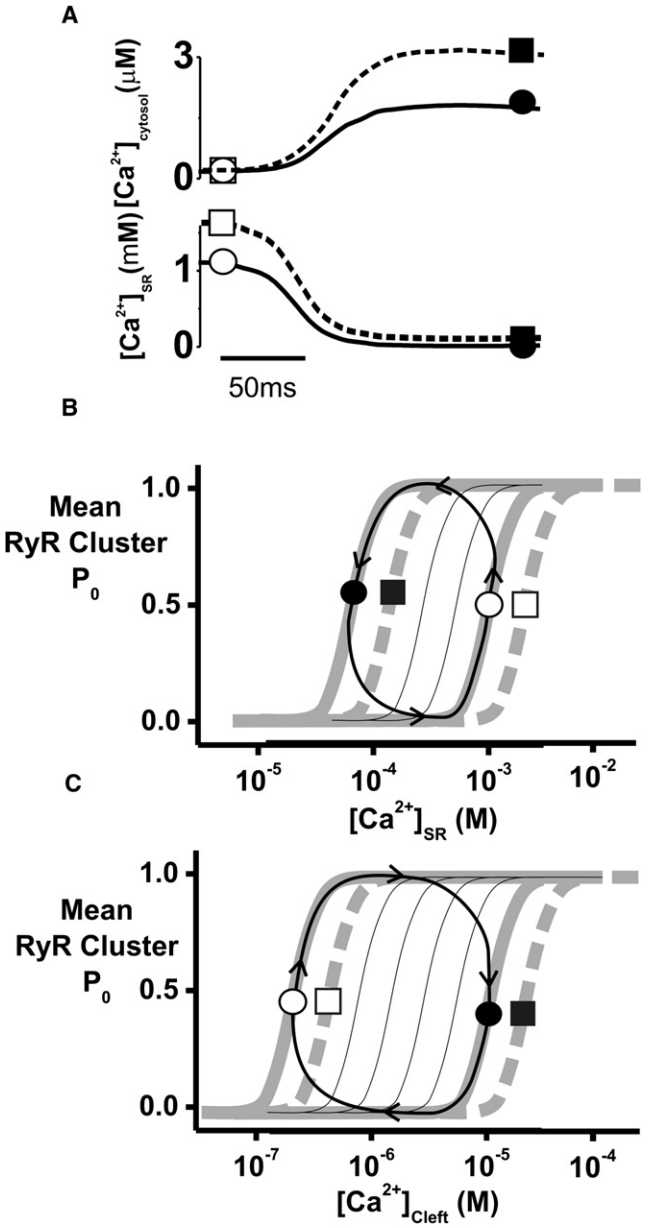

FIGURE 7 Proposed cytosolic and intra-SR RyR Ca^{2+} sensitivity during a Ca^{2+} wave. (*A*) Time course of cytosolic (*upper*) and intra-SR (*lower*) [Ca^{2+}] during a simulated Ca^{2+} wave. Open and solid circles indicate points on curves in *B* and *C*. (*B*) A series of curves showing the sensitivity of RyR. Increasing cytosolic Ca^{2+} shifts the curve to the left. (*C*) Series of sensitivity curves of RyR; reducing luminal Ca^{2+} shifts the curve to the left.

Effects of altered RyR2 threshold: comparison of experimental and computational results

Pharmacological agents can be used to raise or lower RyR2 sensitivity, thus effectively reducing or increasing the effective threshold [Ca^{2+}] within the SR for spontaneous Ca^{2+} release. In this study, tetracaine was used to investigate whether reduced RyR2 sensitivity can alter the fractional depletion of the SR during a Ca^{2+} wave. Under the conditions of this study, tetracaine (50 μM) resulted in an increased SR Ca^{2+} content of ~30%, but there was no dramatic change in the fractional depletion of the SR during a Ca^{2+} wave. However, the minimum [Ca^{2+}] remaining within the SR was ~2 times higher than in the absence of RyR inhibition. This suggests that tetracaine's action on RyR2 includes the desensitization of the processes that control termination of the release process. This can be envisaged as a parallel rightward shift in the Ca^{2+}-sensitivity curves for both the luminal and cytoplasmic control sites (Fig. 7). This proposal is supported by data on isolated RyR2 channels indicating that tetracaine would increase the [Ca^{2+}] required to reaching a minimal Po value (31). Alternatively, a similar relationship would result from a simple blocking action of tetracaine on RyR2. Activation would require a higher fraction of the RyR2 channels in a cluster to achieve SR release, and to increase the effectiveness of stochastic attrition when SR Ca^{2+} release occurs.

The larger total Ca^{2+} release observed in tetracaine was simply the result of the higher maximal free [Ca^{2+}]. As reported in previous studies (16,17), removal of tetracaine caused a burst of high-frequency Ca^{2+} waves, which returned to normal amplitude and frequency after 3–6 s. Previous studies suggested that this characteristic burst is due to a rapid increase of the sensitivity of RyR2, but Ca^{2+} waves do not return to normal until the SR content has been restored (16,17). This explanation was based on the measurements showing that a Ca^{2+} wave depleted the SR by only ~30%, and therefore it took several release events to return SR content to normal. However, this explanation is not supported by the data provided here, which indicate that SR Ca^{2+} release in a single Ca^{2+} wave effectively empties the SR. To investigate the phenomenon further, a computational model was used to simulate the effects of the addition of tetracaine on the local Ca^{2+} release events, assuming that Ca^{2+} waves transiently deplete the SR of Ca^{2+}. Based on the measurements in this study, addition of tetracaine was simulated by a rapid increase in the threshold for SR Ca^{2+} release from 1.1 mM to 1.6 mM. This resulted in a reduction in the frequency but an increase in amplitude of Ca^{2+} waves. Upon removal of tetracaine, the computational model predicted a burst of high-frequency Ca^{2+} waves. Examination of the underlying Ca^{2+} fluxes showed that the burst resulted from a chronically high cytoplasmic Ca^{2+} that stimulated Ca^{2+} uptake by SERCA and reduced the time taken for the SR to reach the threshold for Ca^{2+} release. Thus, the cause of the burst of Ca^{2+} waves on removal of tetracaine was the result of increased cytoplasmic Ca^{2+}, and not increased intra-SR Ca^{2+} as previously thought.

CONCLUSIONS

In conclusion, the results of this study show that spontaneous Ca^{2+} waves lead to substantial but not complete depletion of the SR, a feature that is more easily resolved using the intra-SR signal derived from Fluo-5N rather than cytosolic Fluo-5F. A proportional level of depletion was also seen when RyR2 sensitivity was lowered with tetracaine. The complex behavior seen upon application and removal of tetracaine can be explained by the combined actions of the cytosolic and luminal Ca^{2+}-sensing sites of RyR2. This persistent high level of depletion leads to the hypothesis that luminal control of the termination of Ca^{2+} release occurs when the SR is almost completely depleted of Ca^{2+}. Thus, global SR Ca^{2+} release may be controlled by the number of release sites and not the degree of depletion at each local release site. Further work is necessary to test this hypothesis.

SUPPORTING MATERIAL

One figure is available at http://www.biophysj.org/biophysj/supplemental/S0006-3495(09)00424-X.

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