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Ca²⁺ diffusion and sarcoplasmic reticulum transport both contribute to [Ca²⁺]_i decline during Ca²⁺ sparks in rat ventricular myocytes

Ana M. Gómez, Heping Cheng, W. Jonathan Lederer and Donald M. Bers*†

*Department of Physiology, University of Maryland, Baltimore, MD 21201 and *Department of Physiology, Loyola University Chicago, Maywood, IL 60153, USA*

1. We sought to evaluate the contribution of the sarcoplasmic reticulum (SR) Ca²⁺ pump (*vs.* diffusion) to the kinetics of [Ca²⁺]_i decline during Ca²⁺ sparks, which are due to spontaneous local SR Ca²⁺ release, in isolated rat ventricular myocytes measured using fluo-3 and laser scanning confocal microscopy.
2. Resting Ca²⁺ sparks were compared before (control) and after the SR Ca²⁺-ATPase was either completely blocked by 5 μM thapsigargin (TG) or stimulated by isoprenaline. Na⁺-Ca²⁺ exchange was blocked using Na⁺-free, Ca²⁺-free solution (0 Na⁺, 0 Ca²⁺) and conditions were arranged so that the SR Ca²⁺ content was the same under all conditions when Ca²⁺ sparks were measured.
3. The control Ca²⁺ spark amplitude (281 ± 13 nM) was not changed by TG (270 ± 21 nM) or isoprenaline (302 ± 10 nM). However, the time constant of [Ca²⁺]_i decline was significantly slower in the presence of TG (29.3 ± 4.3 ms) compared with control (21.6 ± 1.5 ms) and faster with isoprenaline (14.5 ± 0.9 ms), but in all cases was much faster than the global [Ca²⁺]_i decline during a control twitch (177 ± 10 ms).
4. The spatial spread of Ca²⁺ during the Ca²⁺ spark was also influenced by the SR Ca²⁺ pump. The apparent 'space constant' of the Ca²⁺ sparks was longest when the SR Ca²⁺ pump was blocked, intermediate in control and shortest with isoprenaline.
5. We conclude that while Ca²⁺ diffusion from the source of Ca²⁺ release is the dominant process in local [Ca²⁺]_i decline during the Ca²⁺ spark, Ca²⁺ transport by the SR contributes significantly to both the kinetics and spatial distribution of [Ca²⁺]_i during the Ca²⁺ spark.

Ca²⁺ sparks are produced when Ca²⁺ release channels in the sarcoplasmic reticulum (SR) open spontaneously or are triggered to open by a local increase in [Ca²⁺]_i (Cheng, Lederer & Cannell, 1993; Cannell, Cheng & Lederer, 1994; López-López, Shacklock, Balke & Weir, 1994; Cannell, Cheng & Lederer, 1995; López-López, Shacklock, Balke & Weir, 1995). The duration of the Ca²⁺ spark reflects a balance of Ca²⁺ efflux from the SR through the ryanodine receptors (RyR) and several factors that can reduce local [Ca²⁺]_i (Cheng, Cannell & Lederer, 1995). Six factors could contribute to the decline of the Ca²⁺ spark: (1) closure of the SR Ca²⁺ release channel (*i.e.* RyR); (2) Ca²⁺ binding to buffers; (3) Ca²⁺ diffusion away from the release site in free solution or on buffers; (4) Ca²⁺ pumping back into the SR via the SR Ca²⁺-ATPase; (5) Ca²⁺ accumulation by the mitochondria; and (6) Ca²⁺ extrusion from the cell by the Na⁺-Ca²⁺ exchanger and sarcolemmal Ca²⁺-ATPase.

Global elevation of [Ca²⁺]_i, produced by a normal twitch [Ca²⁺]_i transient, arises as Ca²⁺ sparks are recruited by the activation of L-type Ca²⁺ channels (Cannell *et al.* 1994, 1995; López-López *et al.* 1995; Santana, Cheng, Gómez, Cannell & Lederer, 1996). Such global increases in [Ca²⁺]_i remove diffusion as a factor contributing to the fall of [Ca²⁺]_i because the spatial distribution of [Ca²⁺]_i is uniform (Cheng *et al.* 1995). In this situation, the dominant processes contributing to global [Ca²⁺]_i decline are SR Ca²⁺-ATPase activity and the Na⁺-Ca²⁺ exchange (Bassani, Bassani & Bers, 1994). In rat ventricular myocytes, the proportion of Ca²⁺ transported by the SR Ca²⁺ pump *vs.* that by Na⁺-Ca²⁺ exchange during relaxation is about 92% *vs.* 7%, whereas in rabbit ventricular myocytes, owing to slower SR Ca²⁺ pumping and faster Na⁺-Ca²⁺ exchange this is 70% *vs.* 28% (Bassani, Bassani & Bers, 1992, 1994; Negretti, O'Neill & Eisner, 1993). In contrast, the Ca²⁺ spark represents a local

†To whom correspondence should be addressed.

elevation of $[Ca^{2+}]_i$ which is free to diffuse from its site of origin to other regions of the cardiac myocyte where $[Ca^{2+}]_i$ is lower. Thus, the rapid decay of the Ca^{2+} spark (time constant, τ , ~ 25 ms) when compared with the global $[Ca^{2+}]_i$ transient (τ , ~ 200 ms) may arise largely from a diffusional component that only the Ca^{2+} spark has. However, as Ca^{2+} diffuses away from the release site it is subject to transport by the same Ca^{2+} transport systems mentioned above. In this paper we have investigated the role of SR Ca^{2+} -ATPase in the decline of the Ca^{2+} spark. We have made use of the specific inhibitor of SR Ca^{2+} -ATPase thapsigargin (Sagara, Fernandez-Belda, de Meis & Inesi, 1992). We examined Ca^{2+} sparks when SR Ca^{2+} -ATPase was completely inhibited, but without depleting the SR of Ca^{2+} (Bassani, Bassani & Bers, 1993; Bassani, Yuan & Bers, 1995). We also used isoprenaline to stimulate SR Ca^{2+} -ATPase via cyclic AMP and the phosphorylation of phospholamban (Tada, Kirchberger, Repke & Katz, 1974). The results indicate that either blocking or stimulating SR Ca^{2+} -ATPase significantly alters the kinetics of local $[Ca^{2+}]_i$ decline during a Ca^{2+} spark, as well as its spatial spread.

METHODS

Cells

Single ventricular cardiac myocytes were prepared from male, Sprague-Dawley rats (~ 2 months old) by standard methods (Cheng *et al.* 1993; Cannell *et al.* 1995). Briefly, animals were killed by an overdose of sodium pentobarbitone (100 mg kg^{-1} i.p.) after which hearts were quickly removed and perfused using the Langendorff method with a series of physiological solutions, including one that contained collagenase (Worthington, Type II; 1 mg ml^{-1}). Following isolation of single cells, extracellular $[Ca^{2+}]_o$ ($[Ca^{2+}]_o$) was increased to 1.0 mM and the cells were stored at room temperature (20 – 23 °C) until needed.

Measurement of $[Ca^{2+}]_i$

Cells were loaded with the Ca^{2+} indicator fluo-3 either by diffusion of the fluo-3 (100 μM) salt from the voltage-clamp pipette into the cell or by using the acetyoxymethyl (AM) ester derivative of fluo-3. This second method involves dissolving fluo-3 AM (50 μg) in 100 μl of a DMSO-Pluronic F-127 mixture ($4:1$ by weight) and incubating cells in a physiological saline containing 5 μM fluo-3 for 20 min and then allowing 15 min in physiological saline alone for de-esterification. Fluorescence was measured using a confocal microscope (see below) with 488 nm excitation from an argon ion laser (Cheng *et al.* 1993).

Images from the confocal microscope

A laser scanning confocal microscope (MRC 600; Biorad, Cambridge, MA, USA) with a $\times 63$ magnification Zeiss Neofluar lens (numerical aperture, 1.25) was used to acquire fluorescence images. Two kinds of image were obtained, both with a resolution of 0.4 μm (in the horizontal plane) and 0.7 μm (in the vertical plane). Images of the whole cell in the x - y plane at full spatial resolution were obtained at 1 s^{-1} . Line-scan images were obtained at 500 s^{-1} but with resolution only along a single line (Cheng *et al.* 1993); these images were used for all of the kinetic data obtained here. Images were analysed using SOM and COMOS software (Biorad) and IDL (Research Systems Inc., Boulder, CO, USA).

The fluo-3 fluorescence was transformed to $[Ca^{2+}]_i$ by a pseudoratio method (Cheng *et al.* 1993; Cannell *et al.* 1994) using the following equation:

$$[Ca^{2+}]_i = \frac{K_d(F/F_0)}{(K_d/[Ca^{2+}]_{i,rest}) + 1 - (F/F_0)},$$

where K_d is the dissociation constant for fluo-3 (400 nm), F is the fluorescence intensity and F_0 is the intensity at rest at each position determined as the mean fluorescence intensity of the lowest fifty pixels along each time line. The $[Ca^{2+}]_i$ at rest ($[Ca^{2+}]_{i,rest}$) for this F_0 was assumed to be 100 nM.

Solutions

Changes in superfusion solutions were carried out in less than 3 s using a bath equipped for rapid solution change. Three basic solutions were used in our experiments. The normal Tyrode solution (NT) contained (mM): 140 NaCl, 5.4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 Hepes and 10 glucose, pH 7.4 . The Na^+ -free, Ca^{2+} -free (0 Na^+ , 0 Ca^{2+}) solution was the same, except Na^+ was replaced by Li^+ and $CaCl_2$ was replaced by 1 mM EGTA. A Na^+ -free solution (0 Na^+ , 2 Ca^{2+}) had the same composition as NT, but Na^+ was replaced with equimolar Li^+ . Thapsigargin (TG) was added from a 10 mM stock in DMSO to the appropriate solution immediately before use to give a final concentration of 5 μM . Caffeine and isoprenaline were added directly to the appropriate solution to produce final concentrations of 10 mM and 1 μM , respectively. In experiments that used patch clamping to control membrane potential (in whole-cell mode), the pipette-filling solution contained (mM): 60 CsCl, 60 KCl, 5 K_2ATP , 5 $MgATP$, 10 Hepes and 0.10 fluo-3, pH 7.2 . Patch-clamp electrodes were pulled from 1.5 mm filamented thin-walled borosilicate glass capillaries (World Precision Instruments, New Haven, CT, USA). All experiments were carried out at room temperature.

Statistical comparisons were carried out using Student's t test or one-way analysis of variance. $P < 0.05$ was considered significant and n refers to the number of analysed Ca^{2+} sparks.

RESULTS

Protocol to allow assessment of Ca^{2+} sparks with SR Ca^{2+} -ATPase blockade by thapsigargin

To assess the contribution of SR Ca^{2+} -ATPase to the $[Ca^{2+}]_i$ decline during Ca^{2+} sparks we compared control Ca^{2+} sparks with those observed when SR Ca^{2+} -ATPase was completely blocked by TG. Comparison of Ca^{2+} sparks in the absence and presence of SR Ca^{2+} -ATPase requires two fundamental conditions. First, the SR Ca^{2+} -ATPase must be completely inhibited and second, Ca^{2+} sparks of comparable amplitude must be observable in the presence of Ca^{2+} -ATPase blockade. To achieve this we used the experimental strategy developed by Bassani *et al.* (1993, 1994, 1995), where SR Ca^{2+} -ATPase could be completely blocked without significantly changing the SR Ca^{2+} content. The results were obtained as follows.

After steady-state stimulation at 1 Hz, the amplitude of a caffeine (10 mM)-induced $[Ca^{2+}]_i$ transient in 0 Na^+ , 0 Ca^{2+} solution was used to assess the SR Ca^{2+} content. Caffeine was washed away in NT and the cell returned to steady-state stimulation. Then stimulation was stopped and the cell

was superfused in 0 Na⁺, 0 Ca²⁺ solution to abolish Ca²⁺ flux via Na⁺-Ca²⁺ exchange and maintain constant SR Ca²⁺ load during rest. Control Ca²⁺ sparks were acquired and then the cell was exposed to 5 μM TG for 2 min (in 0 Na⁺, 0 Ca²⁺ solution), which was sufficient to block completely the SR Ca²⁺ pump (Bassani *et al.* 1993, and see below). During this time resting [Ca²⁺]_i did not change. After Ca²⁺ pump blockade, Ca²⁺ sparks were again acquired in 0 Na⁺, 0 Ca²⁺ solution for 1–2 min until the SR Ca²⁺ content was assessed by a second caffeine exposure in 0 Na⁺, 0 Ca²⁺ solution.

After 2 min of TG treatment there was no significant decrease in the peak [Ca²⁺]_i during the caffeine-induced contracture (control, 1077 ± 187 nM; TG, 1087 ± 198 nM; see Fig. 1A). While the amplitude of caffeine-induced [Ca²⁺]_i transients in rat ventricular myocytes are generally not affected by the presence or absence of [Na⁺]_o (Bassani *et al.* 1994), 0 Na⁺, 0 Ca²⁺ solution with caffeine was used to assess SR Ca²⁺ content in the present study. The time constant (τ) for decline of the caffeine-induced [Ca²⁺]_i transient was slower when Na⁺-Ca²⁺ exchange was blocked

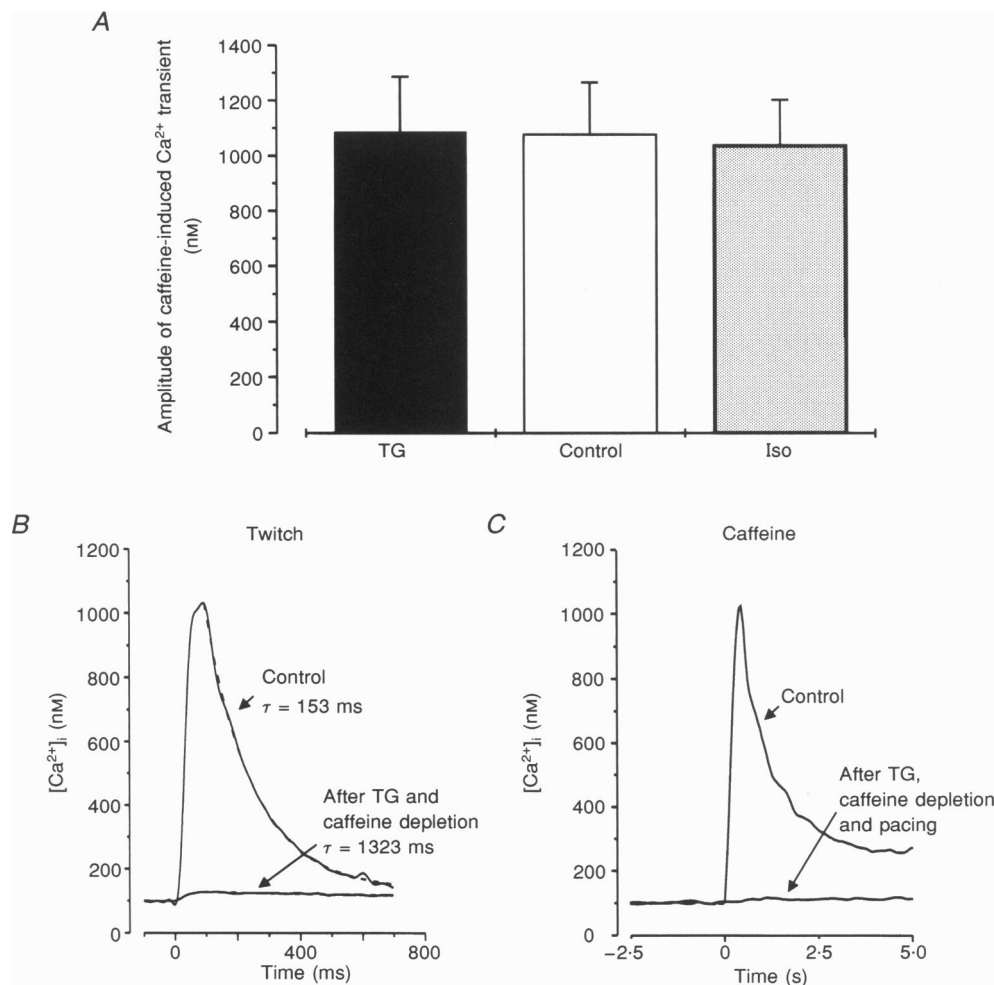


Figure 1. SR Ca²⁺ content and twitch [Ca²⁺]_i transients before and after thapsigargin

A, SR Ca²⁺ content assessed as the amplitude of the [Ca²⁺]_i transient induced by rapid exposure to 10 mM caffeine under the 3 conditions used to measure Ca²⁺ sparks. Thapsigargin (TG) values were obtained after 1 Hz steady-state stimulation and 2 min of rest in 0 Na⁺, 0 Ca²⁺ solution with 5 μM TG (*n* = 6). Isoprenaline (Iso) values were obtained after a series of 5–10 voltage-clamp pulses to load the SR in the presence of 1 μM Iso (*n* = 15). Control values were under similar conditions but without TG or Iso present (*n* = 17). B, steady-state twitch [Ca²⁺]_i transients under control conditions and after exposure first to TG (to inhibit the SR Ca²⁺ pump) and then caffeine (to deplete the SR). Time constants (τ) of [Ca²⁺]_i decline are indicated. C, the peak of the control caffeine exposure (in 0 Na⁺, 0 Ca²⁺ solution) provides an estimate of SR Ca²⁺ content (Bassani *et al.* 1994). The lower trace shows a caffeine exposure after TG application, caffeine-induced depletion and pacing at 1 Hz in an attempt to reload the SR. The lack of an observable [Ca²⁺]_i transient demonstrates effective blockade of SR Ca²⁺ uptake by TG.

(1.7 ± 0.4 s in NT vs. 8.9 ± 3.2 s in 0 Na^+ , 0 Ca^{2+} solution), in agreement with the results of Bassani *et al.* (1994).

The second caffeine exposure after acquisition of Ca^{2+} sparks in TG caused depletion of SR Ca^{2+} content and reduced the amplitude of subsequent twitch $[\text{Ca}^{2+}]_i$ transients by $\sim 90\%$ (Fig. 1B). Figure 1C shows that TG completely prevented reloading of the SR as assessed by a third caffeine exposure (after TG exposure, caffeine-induced SR Ca^{2+} depletion and extensive twitches in an effort to reload). This confirms that the TG concentration used here was sufficient to completely prevent SR Ca^{2+} uptake. In addition, the τ of $[\text{Ca}^{2+}]_i$ decline during the twitch was increased by TG from 177 ± 10 to 1285 ± 215 ms, similar to previous results where SR Ca^{2+} uptake was inhibited by either TG or caffeine (Bassani *et al.* 1994). Thus, this protocol has allowed acquisition of Ca^{2+} sparks at comparable SR Ca^{2+} content in both the presence and absence of SR Ca^{2+} pump function.

Protocol to measure Ca^{2+} sparks in the presence of SR Ca^{2+} pump stimulation by isoprenaline

An additional group of cells was studied when the SR Ca^{2+} pump was stimulated by isoprenaline ($1 \mu\text{M}$). In this series, cells were voltage clamped and loaded with fluo-3 via the patch pipette. After a series of five to ten conditioning pulses (from -80 to 0 mV for 200 ms at 0.03 Hz in 0 Na^+ , 2 Ca^{2+} solution) either Ca^{2+} sparks were measured or caffeine was applied (in 0 Na^+ , 0 Ca^{2+} solution) to assess the SR Ca^{2+} load. This sequence was repeated after exposure to $1 \mu\text{M}$ isoprenaline for 10 min. In the presence of isoprenaline, the pulse protocol was modified so that the steady-state SR Ca^{2+} load was comparable to that for control (steps were to only -10 mV). The amplitude of caffeine-induced $[\text{Ca}^{2+}]_i$ transients in isoprenaline was not significantly different from control levels (control, 1077 ± 188 nM; isoprenaline, 1040 ± 161 nM; Fig. 1A).

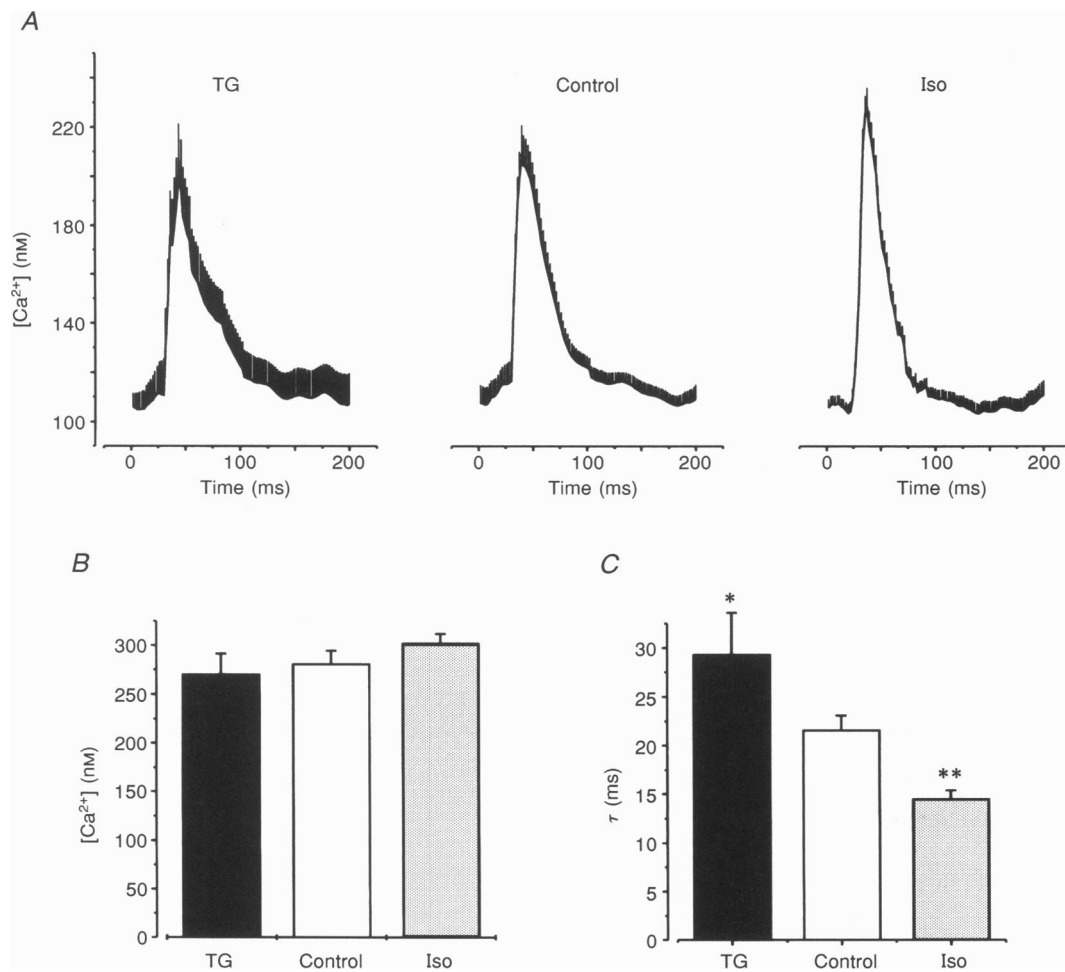


Figure 2. Ca^{2+} spark characteristics after SR Ca^{2+} pump blockade or stimulation

A, signal-averaged Ca^{2+} sparks in the 3 experimental conditions. Sparks were temporally aligned by matching their initial rising phase and the vertical lines indicate ± 1 s.e.m. ($n = 10$, 27 and 86 Ca^{2+} sparks, respectively). B, mean amplitude of Ca^{2+} sparks and C, mean time constant of Ca^{2+} spark decline under the 3 conditions. * $P < 0.05$ and ** $P < 0.001$, significantly different from control conditions.

Ca²⁺ sparks with thapsigargin and isoprenaline

The amplitude and kinetics of control Ca²⁺ sparks were statistically indistinguishable between the control series for the TG and isoprenaline experiments above. Thus, the control sparks were pooled for further analysis. Figure 2A shows signal-averaged Ca²⁺ sparks for control, TG and isoprenaline experiments. Figure 2B shows that the amplitude of Ca²⁺ sparks was not significantly altered by either TG or isoprenaline in these experiments (control, 281 ± 13 nM; TG, 270 ± 21 nM; isoprenaline, 302 ± 10 nM; n = 27, 10 and 86 Ca²⁺ sparks from 8, 5 and 6 cells, respectively). This agrees with the similar SR Ca²⁺ load above and observations of a stereotypical Ca²⁺ spark amplitude (Cheng *et al.* 1993). Isoprenaline increased the frequency of Ca²⁺ sparks (from 0.63 to 1.9 s⁻¹), despite an unchanged SR Ca²⁺ load.

Figure 2C shows that the τ of [Ca²⁺]_i decline during Ca²⁺ sparks was different among the three groups. The τ was longest after TG (29.3 ± 4.3 ms), shortest in isoprenaline (14.5 ± 0.9 ms) and intermediate in control conditions (21.6 ± 1.5 ms). This indicates that the SR Ca²⁺-ATPase contributes significantly to the [Ca²⁺]_i decline of the normal Ca²⁺ spark.

Spatial spread of Ca²⁺ sparks with thapsigargin and isoprenaline

The slower local [Ca²⁺]_i decline observed with SR Ca²⁺ pump blockade might also result in a greater spatial spread, since the SR Ca²⁺ pumps are expected to be broadly distributed throughout the cell. Figure 3A shows the time and width dependence of [Ca²⁺]_i during the averaged control Ca²⁺ spark measured in line-scan mode along the transverse axis

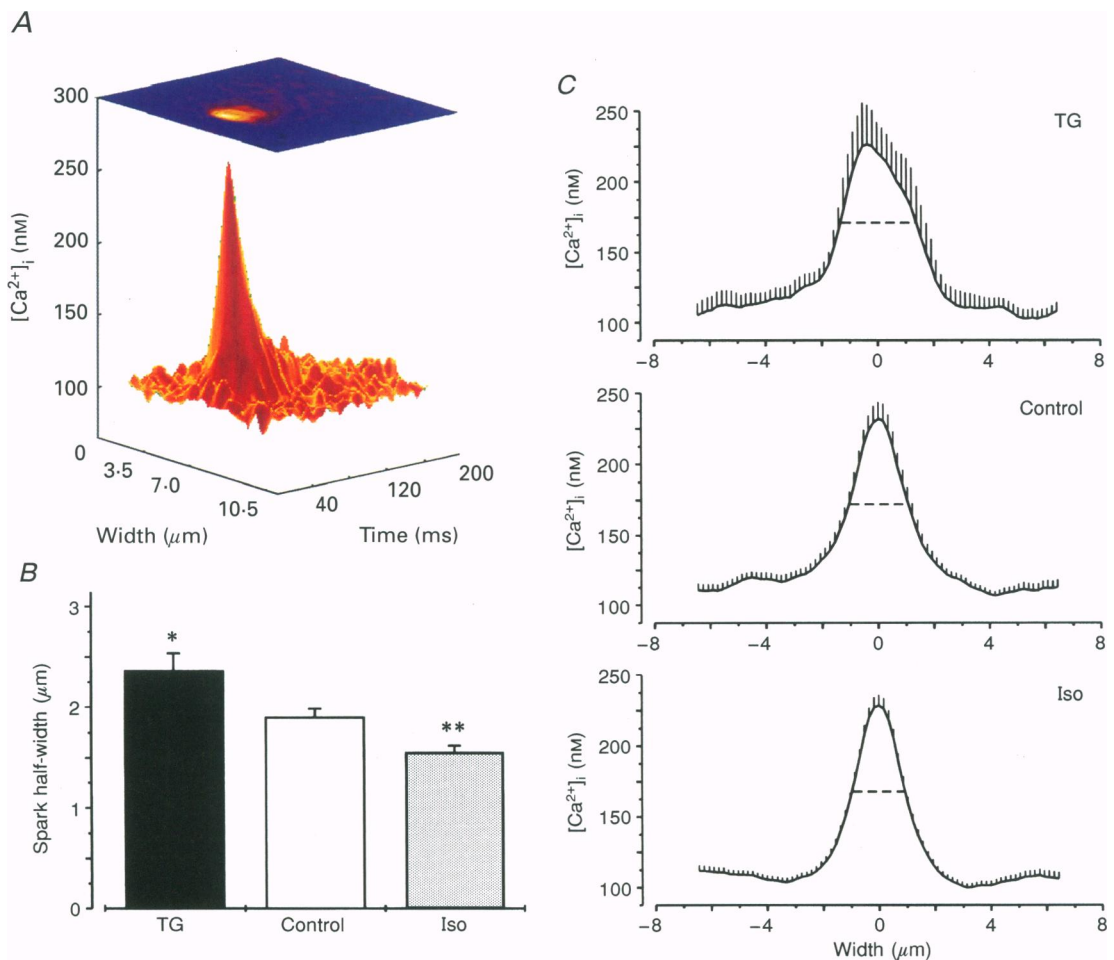


Figure 3. Spatial spread of Ca²⁺ sparks after SR Ca²⁺ pump blockade or stimulation

A, 3-D plot of local [Ca²⁺]_i during a control line scan as a function of time and distance along the transverse scan line. B, mean values of the Ca²⁺ spark width at half-maximal amplitude under the 3 experimental conditions. * P < 0.05 and ** P < 0.01, significantly different from control conditions. C, signal-averaged Ca²⁺ spark widths in the 3 experimental conditions. Sparks were aligned by placing their maximal value at x = 0 and the vertical lines indicate + 1 s.e.m. (n = 10, 27 and 86, respectively). The dashed horizontal lines indicate the full width at half-maximal amplitude.

of the cell. The Ca^{2+} spark spreads in both directions along the line being scanned without obvious asymmetry. While the precise spatial resolution along the length of the scan line is somewhat limiting, the full-width half-height of the Ca^{2+} sparks in the three conditions was significantly different (see Fig. 3B and C). The spatial spread of the Ca^{2+} sparks was largest with TG ($2.4 \pm 0.19 \mu\text{m}$), shortest in isoprenaline ($1.56 \pm 0.07 \mu\text{m}$) and intermediate in control conditions ($1.9 \pm 0.09 \mu\text{m}$). Thus the SR Ca^{2+} -ATPase function modulates both the spatial as well as temporal spread of Ca^{2+} sparks.

DISCUSSION

Ca^{2+} sparks appear to represent relatively stereotypical elementary events of SR Ca^{2+} release both during excitation-contraction coupling and at rest (Cheng *et al.* 1993; Cannell *et al.* 1994, 1995; López-López *et al.* 1995). Furthermore, the apparently homogeneous global $[\text{Ca}^{2+}]_i$ transient during a twitch probably reflects the temporal and spatial summation of many individual Ca^{2+} sparks triggered and synchronized by depolarization and consequent Ca^{2+} influx. Indeed, most of the L-type Ca^{2+} channels must be blocked so that spatially distinct Ca^{2+} sparks can be observed upon activation of the Ca^{2+} current. It is still unclear how many individual SR Ca^{2+} release channels contribute to the functional unit during an individual Ca^{2+} spark (e.g. see Cannell *et al.* 1994; Tsugorka, Rio & Blatter, 1995; Klein, Cheng, Santana, Jiang, Lederer & Schneider, 1996; Lipp & Niggli, 1996). Results in the present study also illustrate that characteristic Ca^{2+} sparks occur in the complete absence of $[\text{Ca}^{2+}]_o$ (with EGTA), indicating that Ca^{2+} influx is not essential for triggering the characteristic Ca^{2+} spark.

Since the SR Ca^{2+} content and the Ca^{2+} spark amplitude were not different, this helps to rule out potential complications due to (1) intrinsic amplitude effects on τ (Bers & Berlin, 1995), (2) slowed kinetics from Ca^{2+} sparks which originate from out of the plane of focus, or (3) fluorescence obtained from outside the nominal voxel space. Nevertheless, these sort of technical constraints place some limitations on the precise quantitative interpretation. Thus we restricted our analysis to semiquantitative terms.

As a first approximation, the rate constants (k) of $[\text{Ca}^{2+}]_i$ decline due to SR Ca^{2+} -ATPase activity and diffusion during a Ca^{2+} spark could be considered to be additive allowing some initial comparisons to be made (where $k = 1/\tau$ from data in Fig. 2C). Thus, the rate constant for $[\text{Ca}^{2+}]_i$ decline due to diffusion only (k_D , with TG in 0 Na^+ , 0 Ca^{2+} solution) is 34.1 s^{-1} and that for both diffusion and the SR Ca^{2+} -ATPase activity ($k_D + k_{\text{SR}}$) is 46.3 s^{-1} . From these numbers it can be inferred that diffusion would account for 74% of the rate of $[\text{Ca}^{2+}]_i$ decline and SR Ca^{2+} -ATPase activity for 26%. When SR Ca^{2+} -ATPase is stimulated by isoprenaline ($k_D + k_{\text{SR,iso}} = 69.0 \text{ s}^{-1}$) this simplified analysis would indicate that the balance is

changed to about 50% due to diffusion and 50% due to the stimulated SR Ca^{2+} -ATPase. This is obviously an oversimplification since the above would imply that k_{SR} is $\sim 12 \text{ s}^{-1}$, whereas the value based on global $[\text{Ca}^{2+}]_i$ decline would give a rate constant of only about 6 s^{-1} (i.e. $\tau = 177 \text{ ms}$ for $[\text{Ca}^{2+}]_i$ decline during a twitch).

Another simplified quantitative consideration uses the number of Ca^{2+} ions released during a Ca^{2+} spark based on an SR Ca^{2+} release flux of 2 pA for 5 ms (31 191 Ca^{2+} ions; Tinker, Lindsey & Williams, 1993). In a $2 \mu\text{m}$ diameter sphere this would be sufficient to raise local total $[\text{Ca}^{2+}]$ by $12.3 \mu\text{M}$ or local mean free $[\text{Ca}^{2+}]$ by 123 nM (assuming 100:1 Ca^{2+} buffering; Hove-Madsen & Bers, 1993). This is comparable to the Ca^{2+} spark. The number of Ca^{2+} ions transported by SR Ca^{2+} -ATPase within this same sphere can also be calculated using the SR Ca^{2+} -pump function estimated by Bassani *et al.* (1994) in intact rat myocytes ($208 \mu\text{M s}^{-1}/(1 + (184 \text{ nM}/[\text{Ca}^{2+}])^{3.9})$). The $[\text{Ca}^{2+}]_i$ which serves as the driving function (in time and space) was taken as an exponentially declining function of time (τ , 20 ms) with a Gaussian spatial profile (full width at half-maximum, $2 \mu\text{m}$) analogous to Fig. 3A. Numerical integration of this Ca^{2+} pump flux yields a transport of 6616 ions or 21% of the total Ca^{2+} ions released as mentioned above. More precise mechanistic calculations would require a detailed spatiotemporal model of Ca^{2+} movements (including diffusion, buffers, pumps and leaks) beyond the scope of the present work.

We have used thapsigargin to block completely SR Ca^{2+} -ATPase during Ca^{2+} sparks. The Na^+ - Ca^{2+} exchange did not contribute to $[\text{Ca}^{2+}]_i$ decline during the Ca^{2+} sparks in this study since they were measured in 0 Na^+ , 0 Ca^{2+} solution. However, Na^+ - Ca^{2+} exchange participation during control Ca^{2+} sparks seems unlikely because $[\text{Ca}^{2+}]_i$ decline during control Ca^{2+} sparks in 0 Na^+ , 0 Ca^{2+} solution were similar to those in normal Na^+ - and Ca^{2+} -containing solution (i.e. τ , $19.6 \pm 1.6 \text{ s}$, $n = 20$, not significantly different from control 0 Na^+ , 0 Ca^{2+} solution). This is not surprising since the Na^+ - Ca^{2+} exchange is about ten times slower than SR Ca^{2+} -ATPase in rat ventricular myocytes (Bassani *et al.* 1992, 1994). Thus, the effect of Na^+ - Ca^{2+} exchange on Ca^{2+} sparks ought to be an order of magnitude smaller than attributed here to SR Ca^{2+} -ATPase. If Ca^{2+} released during a Ca^{2+} spark has preferential access to the Na^+ - Ca^{2+} exchanger rather than the SR Ca^{2+} pump, the effect could be greater.

Since the SR Ca^{2+} -ATPase and mitochondrial Ca^{2+} transport are both more than ten times slower than the Na^+ - Ca^{2+} exchange (Bassani *et al.* 1992, 1994), these systems are not likely to contribute quantitatively to the decline of $[\text{Ca}^{2+}]_i$ during Ca^{2+} sparks. Slow cytosolic Ca^{2+} buffering could also contribute to the decline of $[\text{Ca}^{2+}]_i$ during Ca^{2+} sparks (e.g. at sites from which Mg^{2+} or protons might first have to dissociate before Ca^{2+} can bind), but assessment of this contribution would again require more detailed data and modelling. At higher temperature it might

be expected that the SR Ca²⁺ pump rate would increase to a greater extent than diffusion, so the SR Ca²⁺ pump would probably contribute at least as much to [Ca²⁺]_i decline during Ca²⁺ sparks at 37 °C as described here.

Isoprenaline decreases the sphere of influence of a given Ca²⁺ spark, such that SR Ca²⁺ release from one area will be less likely to induce Ca²⁺ release from a neighbouring region. In principle this aspect of SR Ca²⁺ pump stimulation would tend to decrease the propagation of Ca²⁺ waves. From a practical standpoint, however, this effect will be counterbalanced by the fact that isoprenaline stimulates SR Ca²⁺-ATPase and load which would increase the likelihood of Ca²⁺ sparks or Ca²⁺ waves. Conversely, decreased SR Ca²⁺-ATPase activity would increase the spread of a given Ca²⁺ spark.

- BASSANI, J. W. M., BASSANI, R. A. & BERS, D. M. (1993). Twitch-dependent SR Ca accumulation and release in rabbit ventricular myocytes. *American Journal of Physiology* **265**, C533–540.
- BASSANI, J. W. M., BASSANI, R. A. & BERS, D. M. (1994). Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms. *Journal of Physiology* **476**, 279–293.
- BASSANI, J. W. M., YUAN, Y. & BERS, D. M. (1995). Fractional SR Ca release is altered by trigger Ca and SR Ca content in cardiac myocytes. *American Journal of Physiology* **268**, C1313–1319.
- BASSANI, R. A., BASSANI, J. W. M. & BERS, D. M. (1992). Mitochondrial and sarcolemmal Ca²⁺ transport can reduce [Ca²⁺]_i during caffeine contractures in rabbit cardiac myocytes. *Journal of Physiology* **453**, 591–608.
- BERS, D. M. & BERLIN, J. R. (1995). The kinetics of [Ca]_i decline in cardiac myocytes depends on peak [Ca]_i. *American Journal of Physiology* **268**, C271–277.
- CANNELL, M. B., CHENG, H. & LEDERER, W. J. (1994). Spatial non-uniformities in [Ca²⁺]_i during excitation–contraction coupling in cardiac myocytes. *Biophysical Journal* **67**, 1942–1956.
- CANNELL, M. B., CHENG, H. & LEDERER, W. J. (1995). The control of calcium release in heart muscle. *Science* **268**, 1045–1049.
- CHENG, H., CANNELL, M. B. & LEDERER, W. J. (1995). Partial inhibition of Ca²⁺ current by methoxyverapamil (D600) reveals spatial nonuniformities in [Ca²⁺]_i during excitation–contraction coupling in cardiac myocytes. *Circulation Research* **76**, 236–241.
- CHENG, H., LEDERER, W. J. & CANNELL, M. B. (1993). Calcium sparks: elementary events underlying excitation–contraction coupling in heart muscle. *Science* **262**, 740–744.
- HØVE-MADSEN, L. & BERS, D. M. (1993). Passive Ca buffering and SR Ca uptake in permeabilized rabbit ventricular myocytes. *American Journal of Physiology* **264**, C677–686.
- KLEIN, M. G., CHENG, H., SANTANA, L. F., JIANG, Y.-H., LEDERER, W. J. & SCHNEIDER, M. F. (1996). Two mechanisms of quantized calcium release in skeletal muscle. *Nature* **379**, 455–458.
- LIPP, P. & NIGGLI, E. (1996). Submicroscopic calcium signals as fundamental events of excitation–contraction coupling in guinea-pig cardiac myocytes. *Journal of Physiology* **492**, 31–38.
- LÓPEZ-LÓPEZ, J. R., SHACKLOCK, P. S., BALKE, C. W. & WIER, W. G. (1994). Local, stochastic release of Ca²⁺ in voltage-clamped rat heart cells: visualization with confocal microscopy. *Journal of Physiology* **480**, 21–29.
- LÓPEZ-LÓPEZ, J. R., SHACKLOCK, P. S., BALKE, C. W. & WIER, W. G. (1995). Local calcium transients triggered by single L-type calcium channel currents in cardiac cells. *Science* **268**, 1042–1045.
- NEGRETTI, N., O'NEILL, S. C. & EISNER, D. A. (1993). The relative contributions of different intracellular and sarcolemmal systems to relaxation in rat ventricular myocytes. *Cardiovascular Research* **27**, 1826–1830.
- SAGARA, Y., FERNANDEZ-BELDA, F., DE MEIS, L. & INESI, G. (1992). Characterization of the inhibition of intracellular Ca transport ATPases by thapsigargin. *Journal of Biological Chemistry* **267**, 12606–12613.
- SANTANA, L. F., CHENG, H., GÓMEZ, A. M., CANNELL, M. B. & LEDERER, W. J. (1996). Relation between the sarcolemma Ca²⁺ current and Ca²⁺ sparks and local control theories for cardiac excitation–contraction coupling. *Circulation Research* **78**, 166–171.
- TADA, M., KIRCHBERGER, M. A., REPKE, D. I. & KATZ, A. M. (1974). The stimulation of calcium transport in cardiac sarcoplasmic reticulum by adenosine 3':5'-monophosphate-dependent protein kinase. *Journal of Biological Chemistry* **249**, 6174–6180.
- TINKER, A., LINDSEY, A. R. G. & WILLIAMS, A. J. (1993). Cation conductance in the calcium release channel of cardiac sarcoplasmic reticulum under physiological and pathophysiological conditions. *Cardiovascular Research* **27**, 1820–1825.
- TSUGORKA, A., RIOS, E. & BLATTER, L. A. (1995). Imaging elementary events of calcium release in skeletal muscle cells. *Science* **269**, 1723–1726.

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Author's present address

H. Cheng: Laboratory of Cardiovascular Sciences, National Institute on Aging, Baltimore, MD 21224, USA.

Author's email address

D. M. Bers: dbers@luc.edu

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