The role of luminal Ca^{2+} in the generation of Ca^{2+} waves in rat ventricular myocytes

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1. We used confocal Ca^{2+} imaging and fluo-3 to investigate the transition of localized Ca^{2+} releases induced by focal caffeine stimulation into propagating Ca^{2+} waves in isolated rat ventricular myocytes.

2. Self-sustaining Ca^{2+} waves could be initiated when the cellular Ca^{2+} load was increased by elevating the extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) and they could also be initiated at normal Ca^{2+} loads when the sensitivity of the release sites to cytosolic Ca^{2+} was enhanced by low doses of caffeine. When we prevented the accumulation of extra Ca^{2+} in the luminal compartment of the sarcoplasmic reticulum (SR) with thapsigargin, focal caffeine pulses failed to trigger self-sustaining Ca^{2+} waves on elevation of $[Ca^{2+}]_o$. Inhibition of SR Ca^{2+} uptake by thapsigargin in cells already preloaded with Ca^{2+} above normal levels did not prevent local Ca^{2+} elevations from triggering propagating waves. Moreover, wave velocity increased by 20 %. Tetracaine (0·75 mM) caused transient complete inhibition of both local and propagating Ca^{2+} signals, followed by full recovery of the responses due to increased SR Ca^{2+} accumulation.

3. Computer simulations using a numerical model with spatially distinct Ca^{2+} release sites suggested that increased amounts of releasable Ca^{2+} might not be sufficient to generate self-sustaining Ca^{2+} waves under conditions of Ca^{2+} overload unless the threshold of release site Ca^{2+} activation was set at relatively low levels ($< 1{\cdot}5\ \mu$M).

4. We conclude that the potentiation of SR Ca^{2+} release channels by luminal Ca^{2+} is an important factor in Ca^{2+} wave generation. Wave propagation does not require the translocation of Ca^{2+} from the spreading wave front into the SR. Instead, it relies on luminal Ca^{2+} sensitizing Ca^{2+} release channels to cytosolic Ca^{2+}.

In mammalian cardiac myocytes, contractile activation occurs in response to Ca^{2+} influx from the extracellular space, triggering Ca^{2+} release via Ca^{2+}-sensitive channels located in the membrane of the sarcoplasmic reticulum (SR) (Bers, 1991; Stern & Lakatta, 1992; Eisner *et al.* 1998). Despite its self-regenerating nature, this process of Ca^{2+}-induced Ca^{2+} release (CICR) in myocytes is not normally all-or-none but instead is smoothly graded according to the amounts of trigger Ca^{2+} entering into the cell (Cannell *et al.* 1987; Beuckelmann & Wier, 1988). Under certain conditions, such as increased cellular Ca^{2+} load, Ca^{2+} release does show signs of uncontrollable behaviour. Spontaneous discharges of SR Ca^{2+} begin locally, then propagate as regenerative Ca^{2+} waves through the cell (Kort *et al.* 1985; Wier *et al.* 1987; Takamatsu & Wier, 1990; Lipp & Niggli, 1994; Trafford *et al.* 1995; Engel *et al.* 1995; Wussling & Salz, 1996; Cheng *et al.* 1996; Lukyanenko *et al.* 1996). This chaotic behaviour has been implicated in certain cardiac abnormalities including after-depolarizations and Ca^{2+}-dependent arrhythmias (Ishide, 1996). At the present time, the factors responsible for preventing CICR from self regenerating or the alterations responsible for transition from graded release to all-or-none spontaneous release are not precisely understood.

The clue to understanding these questions may come from the recognition that the control of CICR occurs in spatially discrete sub-domains (Niggli & Lederer, 1990; Stern, 1992; Györke & Palade, 1993). Local fluorescence signals reflecting activation of individual release sites have been visualized directly using confocal imaging (Cheng *et al.* 1993; Lipp & Niggli, 1994). These local Ca^{2+} signals, called Ca^{2+} sparks, are confined to an area with a diameter of $< 3\ \mu$m and reach a peak amplitude of 100–500 nM. It appears that Ca^{2+} release during both normal excitation–contraction coupling and propagating Ca^{2+} waves is a result of spatial and temporal summation of Ca^{2+} sparks (Cannell *et al.* 1994; Lopez-Lopez *et al.* 1995; Cheng *et al.* 1996). Under normal conditions, almost all sparks remain localized and die out without inducing release in adjacent release sites (Cheng *et*

al. 1993, 1996; Lukyanenko *et al.* 1996). Under conditions of increased cellular Ca^{2+} load, sparks increase in amplitude and frequency and become initiation sites of propagating Ca^{2+} waves (Cheng *et al.* 1993, 1996; Lukyanenko *et al.* 1996; Satoh *et al.* 1997).

In principle, propagation of Ca^{2+} release could be caused by any of the following mechanisms. (1) Ca^{2+} acting at the cytoplasmic activation site of the SR Ca^{2+} release channel in a manner similar to that during normal excitation–contraction coupling (Stern *et al.* 1988; Stern, 1992; Trafford *et al.* 1995). Transition of local release to propagating release could be due to a combination of increased resting Ca^{2+} and a larger amount of Ca^{2+} released during a spark. (2) Ca^{2+} acting at an intraluminal site on the SR Ca^{2+} release channel following its uptake into the SR (Takamatsu & Wier, 1990; Fabiato, 1992). This possibility is consistent with recent evidence showing that increased luminal Ca^{2+} can activate the Ca^{2+} release channels in lipid bilayers (Sitsapesan & Williams, 1994; Lukyanenko *et al.* 1996). It is also consistent with results indicating that increased SR Ca^{2+} load can potentiate CICR release in intact and patch-clamped myocytes (Han *et al.* 1994; Janczewski *et al.* 1994; Bassani *et al.* 1995; Lukyanenko *et al.* 1996; Györke *et al.* 1997; Györke & Györke, 1998). (3) A combination of the first and second mechanisms when the sensitivity of ryanodine receptors (RyRs) to cytosolic Ca^{2+} is enhanced by increased luminal Ca^{2+}. In support of this possibility, increased luminal Ca^{2+} has been shown to increase the sensitivity of RyRs to cytosolic Ca^{2+} in lipid bilayers (Lukyanenko *et al.* 1996; Györke & Györke, 1998).

To distinguish between these possibilities we monitored the subcellular properties of SR Ca^{2+} release using confocal Ca^{2+} imaging and fluo-3 in rat ventricular myocytes. We examined the transition of localized Ca^{2+} release into propagating Ca^{2+} waves under various experimental conditions, including inhibition of the SR Ca^{2+} pump by thapsigargin, sensitization of the SR Ca^{2+} release channels to cytosolic Ca^{2+} by caffeine, and inhibition of the SR Ca^{2+} channels by tetracaine. In addition we investigated the factors for successful Ca^{2+} wave propagation using a numerical model of the Ca^{2+} wave. Our results indicate that the primary factor responsible for propagation of the Ca^{2+} wave is cytosolic Ca^{2+}. Luminal Ca^{2+} plays a modulatory role by enhancing the sensitivity of the Ca^{2+} release channels to cytoplasmic Ca^{2+}.

METHODS

Cell isolation and experimental solutions

Adult Sprague–Dawley rats (200–300 g) were killed by lethal injection of pentobarbitone sodium (Nembutal; 100 mg kg^{-1} I.P.) as approved by the Animal Care and Use Committee. Single ventricular myocytes were obtained by enzymatic dissociation as described before (Györke *et al.* 1997). The cells were loaded with fluo-3 by a 20 min incubation with 5 μM fluo-3 AM (acetoxymethyl ester form; Molecular Probes) at 23 °C.

The Tyrode solution contained (mM): 140 NaCl, 2 KCl, 0·5 $MgCl_2$, 1–5 $CaCl_2$, 10 Hepes, 0·25 NaH_2PO_4, 5·6 glucose, pH 7·3. Tetrodotoxin (Sigma) at 10 μM was added to the bathing solution to avoid depolarization-induced Ca^{2+} release due to spontaneous action potentials. Caffeine, thapsigargin (Calbiœchem) or tetracaine were used at the concentrations indicated.

Confocal microscopy

Experiments were performed using an Olympus laser scanning confocal microscope (LSM-GB200) equipped with an Olympus ×60, 1·4 NA objective (Lukyanenko *et al.* 1996). Fluo-3 was excited by light at 488 nm (25 mW argon laser, intensity attenuated to 1–3 %), and fluorescence was measured at wavelengths of >515 nm (confocal detector aperture set to 25 % of maximum). As determined with 0·1 μm fluorescent beads (Molecular Probes), the system provided an axial resolution of 0·4 μm and a depth of field of about 0·7 μm. Images were acquired in the linescan mode at a rate of 8 or 32 milliseconds per scan. For calibration purposes, the total linescan $[Ca^{2+}]$ in 1 mM $[Ca^{2+}]_o$ was assumed to be 100 nM and it served as a reference point for the determination of $[Ca^{2+}]$ in the course of an experiment. $[Ca^{2+}]$ was calculated from fluo-3 fluorescence using an equation and calibration parameters described previously (Cheng *et al.* 1993). Image processing and analysis were performed by using NIH Image (NIH, Bethesda, MD, USA) and IDL software (Research Systems Inc., Boulder, CO, USA).

Lipid bilayer experiments

Heavy sarcoplasmic reticulum (SR) microsomes were isolated by differential centrifugation from the ventricles of dog heart (Dettbarn *et al.* 1994). The dogs were killed by lethal injection of Nembutal (70 mg kg^{-1} I.V.) as approved by the Animal Care and Use Committee. SR microsomes were fused into planar lipid bilayers and single channels were recorded as described previously (Lukyanenko *et al.* 1996). SR vesicles were added to one side of the bilayer (defined as *cis*) and the other side was defined as *trans* (virtual ground). Bilayers contained 80 % phosphatidylethanolamine and 20 % phosphatidylcholine dissolved in decane at a final concentration of 50 mg ml^{-1}. Standard solutions contained 350 mM *cis* $CsCH_3SO_3$, 20 mM *trans* $CsCH_3SO_3$, 20 μM $CaCl_2$, 20 mM Hepes (pH 7·4). After channel incorporation, the *trans* $CsCH_3SO_3$ was adjusted to 350 mM. Single channel recording was carried out using an Axopatch 200A (Axon Instruments) patch-clamp amplifier. Data were filtered at 2 kHz and digitized at 5 kHz. Acquisition and analysis of data were performed using pCLAMP 6.01 software (Axon Instruments).

Model of the Ca^{2+} wave

To simulate Ca^{2+} waves, we used a numerical model of CICR with spatially discrete release sites. The model includes 50 individual release sites (equally spaced at intervals of 2 μm), two spatially homogeneous removal fluxes (SR Ca^{2+} pumps and soluble buffers), diffusible dye, the calcium–dye complex (Ca:dye) and calcium bound to endogenous buffers (Ca:B). The spatiotemporal distributions of the free cytoplasmic Ca^{2+}, the Ca:dye complex and the free dye, along with the temporal evolution of the bound buffers, are described by the following coupled system of reaction–diffusion equations and ordinary differential equation:

$$\partial[Ca^{2+}]/\partial t = D_{Ca}\nabla^2[Ca^{2+}] + q_{rel} - q_{rem} + q_{rel0} \quad (1)$$

$$\partial[Ca{:}dye]/\partial t = D_{Ca:dye}\nabla^2[Ca{:}dye] + q_{dye} \quad (2)$$

$$\partial[dye]/\partial t = D_{dye}\nabla^2[dye] - q_{dye} \quad (3)$$

$$d[Ca{:}B]/dt = q_b. \quad (4)$$

Here D_{Ca}, $D_{Ca:dye}$ and D_{dye} are the diffusion coefficients of Ca^{2+}, Ca:dye and free dye, respectively (the endogenous buffers are assumed to be immobile, see below); $\nabla^2[Ca^{2+}]$, ∇^2[Ca:dye] and ∇^2[dye] are the Laplacians; q_{rel} is the release flux and q_{rem} the removal flux. The removal flux consists of three contributions:

$$q_{rem} = q_b + q_{SR} + q_{dye}. \quad (5)$$

The quantity q_b is the removal by binding to endogenous buffers, and q_{SR} is the removal by the Ca^{2+}-ATPase of the SR. q_{dye} is the binding flux of free calcium to the indicator dye. The quantity q_{rel0} is the calcium release from the SR under basal conditions. Removal by the Ca^{2+}-ATPase and Na^+–Ca^{2+} exchanger of the sarcolemma (SL) is ignored in this study. The Ca^{2+} release flux, q_{rel}, of each release site was implemented using the model equations and parameter values in the appendix of Keizer *et al.* (1998; eqns 7–10). It is given by the following expression:

$$q_{rel}(r,t) = \begin{cases} v_{rel} f_0([Ca^{2+}](r,t))([Ca^{2+}]_{SR} - [Ca^{2+}](r,t)) & \text{if } [Ca^{2+}](r,t) \geqslant c^* \text{ and } r = r_i \\ 0 & \text{otherwise.} \end{cases} \quad (6)$$

Here the coefficient v_{rel} is the conductance of the release site, $f_0([Ca^{2+}](r,t))$ is the calcium-dependent fractional activation of each site and $[Ca^{2+}](r,t)$ is the local free calcium concentration. The quantity c^* is the threshold value of myoplasmic calcium above which the site is able to release calcium. r_i is the location of release site i. $[Ca^{2+}]_{SR}$ is the calcium concentration in the sarcoplasmic reticulum (taken to be constant in a given simulation).

With regard to buffering, the flux was calculated from the following equation, which lumps all the endogenous buffers into a single immobile pool:

$$q_b = k_{b,on}[Ca^{2+}]([B]_0 - [Ca:B]) - k_{b,off}[Ca:B]. \quad (7)$$

The rate constants for binding and dissociation are given by $k_{b,on}$ and $k_{b,off}$ respectively. $[B]_0$ is the total concentration of buffers and [Ca:B] is the concentration of the calcium–buffer complexes. Implicit in this expression is the use of the conservation relation:

$$[B]_0 = [B] + [Ca:B]. \quad (8)$$

Then the concentration of the free buffer at any time, [B], can simply be found by difference. The use of this relation is valid when the initial distribution of the buffers is uniform and binding of calcium to the buffers does not change the diffusion coefficient of the buffers. The flux transported by the Ca^{2+} pumps was calculated from the expression given by Tang & Othmer (1994):

$$q_{SR} = V_{SR}[Ca^{2+}]^4/([K_{SR}]^4 + [Ca^{2+}]^4), \quad (9)$$

where V_{SR} is the maximal capacity and K_{SR} the calcium capacity at which the transport is half-maximal for a particular process. The flux of calcium binding to the indicator is given by the following expression:

$$q_{dye} = k_{dye,on}[Ca^{2+}][dye] - k_{dye,off}[Ca:dye]. \quad (10)$$

As with the endogenous buffers, a conservation relation among the total dye, bound dye and free dye may be employed when the initial distribution of dye is uniform and the diffusion coefficients of the free and bound dye are the same. However, we chose in this case to model the free dye in an equivalent fashion with a reaction diffusion equation instead of the conservation relations because we wished to incorporate the flexibility to relax the assumptions that permit the conservation relation in future studies.

The term q_{rel0} is given by:

$$q_{rel0} = V_{SR}[Ca^{2+}]_0^4/([K]_{SR}^4 + [Ca^{2+}]_0^4). \quad (11)$$

Table 1. Parameters of the Ca^{2+} wave model

Parameter	Value	Source
D_{Ca} (μm^2 ms^{-1})	0·30	Albritton *et al.* (1992)
$D_{Ca:dye}$ (μm^2 ms^{-1})	0·02	Harkins *et al.* (1993)
D_{dye} (μm^2 ms^{-1})	0·02	Harkins *et al.* (1993)
V_{SR} (μM s^{-1})	208	Smith *et al.* (1998)
K_{SR} (nM)	120	Tang & Othmer (1994)
$k_{b,on}$ (nM^{-1} ms^{-1})	10^{-4}	Sipido & Wier (1991)
$k_{b,off}$ (ms^{-1})	4×10^{-2}	Sipido & Wier (1991)
$k_{dye,on}$ (nM^{-1} ms^{-1})	$2{\cdot}4 \times 10^{-4}$	Escobar *et al.* (1995)
$k_{dye,off}$ (ms^{-1})	$1{\cdot}8 \times 10^{-2}$	Escobar *et al.* (1995)
$[B]_0$ (nM)	$1{\cdot}20 \times 10^5$	Fabiato (1983)
$[Ca^{2+}]_{SR}$ (mM)	5	Chen *et al.* (1996)

Definitions are given in the text.

The parameters of release, diffusion, buffering and uptake of Ca^{2+} are presented in Table 1.

The governing equations are subject to the following initial and boundary conditions:

$$[Ca^{2+}](r,0) = [Ca^{2+}](0,t) = [Ca^{2+}](a,t) = [Ca^{2+}]_0$$

$$[Ca:dye](r,0) = [Ca:dye](0,t) = [Ca:dye](a,t) = [Ca:dye]_0$$

$$[dye](r,0) = [dye](0,t) = [dye](a,t) = [dye]_0$$

$$[Ca:B](0) = [Ca:B]_0 = \frac{[Ca^{2+}]_0}{(k_{b,off}/k_{b,on}) + [Ca^{2+}]_0}. \quad (12)$$

The value of the basal calcium level was taken to be $[Ca^{2+}]_0 = 0{\cdot}1$ μM. The basal values $[Ca:dye]_0$ and $[dye]_0$ were taken to be in equilibrium with $[Ca^{2+}]_0$ ($[Ca:dye]_0 = 5$ μM, $[dye]_0 = 45$ μM, $[Ca:B]_0 = 0{\cdot}041$ μM). The dimension $a = 100$ μm, and is the radius of the spherical domain being simulated. The coupled reaction–diffusion equations were solved numerically using an explicit Euler scheme. The time derivatives were made discrete using a forward finite difference approximation, and the spatial derivatives were made discrete using a second-order, central finite difference approximation. Our time step was $\Delta t = 8{\cdot}571 \times 10^{-2}$ ms, and our space step was $\Delta x = 0{\cdot}1$ μm. The modelling code was written in VISUAL FORTRAN Standard Edition 5.0.A (Digital) and executed on a Pentium IID 266 desktop computer.

RESULTS

Generation and measurement of Ca^{2+} waves

To study the mechanisms underlying Ca^{2+} wave propagation we used the technique of local caffeine application. It has been demonstrated that Ca^{2+} release by caffeine can be confined to small regions of the cell to trigger Ca^{2+} waves propagating away from the area of application (Trafford *et al.* 1995). Caffeine (10 mM) was applied through a micropipette (~1 μm diameter) positioned near the surface of fluo-3-loaded cells and the resulting $[Ca^{2+}]_i$ changes were measured by repeatedly scanning a single line along the longitudinal axis of the cell. The spatial characteristics of our local solution application system were visualized upon

injection of fluorescein-containing solution into the bath (Fig. 1*Aa*). As can be seen, the solution application was confined within an area of $< 20\ \mu$m diameter. Local application of caffeine to cells induced Ca^{2+} waves which propagated from the area of initiation giving rise to a characteristic inverted 'V' fluorescence pattern on the linescan image (Fig. 1*Ac*).

In accordance with previous studies (Trafford *et al.* 1990, 1995), the potency of the cells to generate propagating responses depended on their Ca^{2+} loading status. With $[Ca^{2+}]_o < 2$ mM, most responses triggered by caffeine typically decayed in both amplitude and propagation velocity, and died out within 20–30 μm of their site of origin. With $[Ca^{2+}]_o > 2$ mM, most responses propagated throughout the entire cell at constant amplitude and velocity. Figure 1*A* shows images from a typical experiment in which abortive waves measured with 1 mM $[Ca^{2+}]_o$ (Fig. 1*Ab*) became regenerative 1 min after increasing $[Ca^{2+}]_o$ to 3 mM (Fig. 1*Ac*). Similar results were obtained in eight out of ten cells in which $[Ca^{2+}]$ was changed from 1 to between 2 and 5 mM. In the two remaining experiments (at 2 mM $[Ca^{2+}]_o$), local caffeine pulses failed to trigger propagating Ca^{2+} waves. Figure 1*B* plots time-dependent fluorescence changes measured from the site of caffeine applications before and after elevating $[Ca^{2+}]_o$. In accordance with our previous studies (Lukyanenko *et al.* 1996), elevating $[Ca^{2+}]_o$ resulted in a slight increase in the resting fluorescence and a larger increase in the amplitude of the peak caffeine-induced fluorescence transients. The position of the Ca^{2+} wave in the x–t plane was defined at half-maximal amplitude of the rising phase of the signal using a computer selection algorithm (Fig. 1*Ad*). The average velocity of the propagating wave was determined by fitting a linear function to the position of the wave (Fig. 1*C*). The average wave velocity varied from 32 to 74 μm s^{-1} (46·0 $\pm$ 2·3 μm s^{-1}, $n = 25$) at $[Ca^{2+}]_o$ of 3 mM. These estimates of the Ca^{2+} wave velocities should not be affected significantly by local shortening of the cell. It has been shown previously that

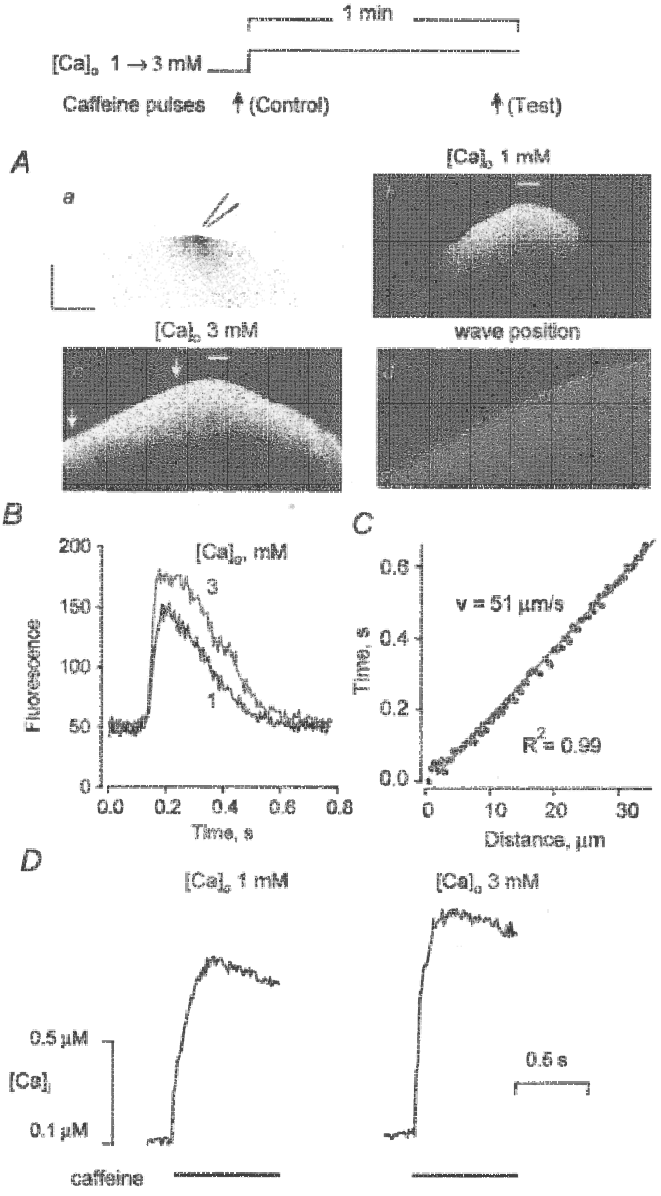

Figure 1. The effects of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ responses to focal caffeine applications

Aa, linescan image illustrating diffusion of fluorescein in water following pressure injection (10 p.s.i. for 20 ms) of fluorescein-containing solution (5 μM l^{-1}) from a micropipette. *b* and *c*, linescan images of fluorescence changes induced by focal application of caffeine (10mM) with injection settings similar to those in *a* at 1 mM $[Ca^{2+}]_o$ (non-propagating response; *b*) and 1 min after increasing $[Ca^{2+}]_o$ to 3 mM (propagating Ca^{2+} wave; *c*). The experimental protocol is presented schematically at the top. *d*, portion of the image in *c* marked by arrows; white dots indicate the position of the wave defined at half-maximal amplitude of the local $[Ca^{2+}]_i$. Calibration bars: horizontal, 14 μm; vertical, 0·28 s. *B*, time-dependent changes in fluorescence at the site of caffeine applications (marked in *Ab* and *c* by bars) at 1 mM $[Ca^{2+}]_o$ (black trace) and 3 mM $[Ca^{2+}]_o$ (grey trace). *C*, linear fit to the position of the wave as defined in *Ad*; the reciprocal of the slope of the line yields the average wave velocity $v = 51\ \mu$m s^{-1}. *D*, space-averaged Ca^{2+} transients elicited by application of 20 mM caffeine to the whole bath before (lefthand trace) and 1 min after increasing $[Ca^{2+}]_o$ from 1 to 3 mM (righthand trace).

contractile waves follow calcium waves with a delay of 50–100 ms (Kort *et al.* 1985). Thus, as has been argued previously (Wussling & Salz, 1996), although regional shortening can affect initial Ca^{2+} wave velocity it should have no impact on the velocity of the Ca^{2+} wave at later times (> 100 ms), when the Ca^{2+} and contractile waves travel at the same speed.

The effects of increasing $[Ca^{2+}]_o$ on SR Ca^{2+} content were assessed in a separate series of experiments, in which caffeine (20 mM) was applied to the whole bath and changes in space-averaged $[Ca^{2+}]$ were measured (Lukyanenko *et al.* 1996). As indexed by changes in the amplitude of the caffeine-induced Ca^{2+} transients (0·84 ± 0·05 μM *vs.* 1·10 ± 0·06 μM, $n = 8$), the SR Ca^{2+} load increased by 26% upon elevating $[Ca^{2+}]_o$ from 1 to 3 mM (Fig. 1*D*). In addition, the resting $[Ca^{2+}]$ tended to increase slightly (from 100 ± 6 to 112 ± 10 nM). These results are in agreement with our previous assessment of changes in SR Ca^{2+} load caused by elevations in extracellular $[Ca^{2+}]$ (Lukyanenko *et al.* 1996).

Effects of thapsigargin

The transition from an abortive Ca^{2+} wave to a regenerative Ca^{2+} wave on elevation of $[Ca^{2+}]_o$ could be caused by increases in either cytosolic or intra-SR $[Ca^{2+}]$. To distinguish between these possibilities, we examined the ability of local caffeine application to induce propagating Ca^{2+} waves under conditions when the SR Ca^{2+} uptake was inhibited by thapsigargin before elevating bathing $[Ca^{2+}]$. Thapsigargin selectively inhibits the SR Ca^{2+}-ATPase (Thastrup *et al.* 1989; Kirby *et al.* 1992). As illustrated in Fig. 2*A*, exposure of the cells to thapsigargin prior to elevation of $[Ca^{2+}]_o$ resulted in a failure of caffeine pulses to trigger regenerative Ca^{2+} waves. In these experiments, thapsigargin (10 μM) was introduced to the bath 1 min before elevating $[Ca^{2+}]$ from 1 to 3 mM and the caffeine pulse was applied 2 min after the Ca^{2+} elevation. At this concentration and exposure time, thapsigargin has been shown to inhibit SR Ca^{2+} uptake by about 90% without causing a significant change in the SR Ca^{2+} load (Bassani *et al.* 1993). At the site of caffeine application the basal fluorescence was again slightly elevated, but the peak amplitude of the caffeine-induced fluorescence signal did not show a significant change in the presence of thapsigargin (Fig. 2*B*). This result would be consistent with the notion that thapsigargin, while preventing uptake of extra Ca^{2+} into the SR, did not cause a measurable loss of Ca^{2+} from the SR during the time period of the measurements. Similar results were obtained in four out of four other experiments.

The effects of thapsigargin on the Ca^{2+} load and Ca^{2+} uptake capacity of the SR were further investigated in a separate series of experiments with caffeine application to the whole bath. Figure 2*C* illustrates caffeine-induced Ca^{2+} transients measured under control conditions and also after successive introductions to the bath of thapsigargin (10 μM) and elevated Ca^{2+} (3 mM). Consistent with the results of experiments employing local caffeine applications (Fig. 2*B*), elevation of $[Ca^{2+}]_o$ in the presence of thapsigargin, while resulting in a small elevation in the basal $[Ca^{2+}]$ (from 100 ± 9 to 140 ± 15 μM, $n = 5$), did not cause a significant increase in amplitude of the caffeine-induced Ca^{2+} transients (0·81 ± 0·09 *vs.* 0·86 ± 0·10 μM). The amplitude of the second caffeine-induced Ca^{2+} transient measured 2 min later in the continuous presence of thapsigargin was reduced dramatically (by 72 ± 11%), verifying a substantial inhibition in SR Ca^{2+} uptake. These results confirm that thapsigargin can effectively prevent the accumulation of extra Ca^{2+} in the SR on increasing $[Ca^{2+}]_o$ under our experimental conditions. Taken together, these results suggest that the increased accumulation of Ca^{2+} in the SR is a critical factor in the generation of propagating Ca^{2+} waves under conditions of increased cellular Ca^{2+} load. The remainder of this study was therefore concerned with defining the mechanisms through which increased $[Ca^{2+}]_{SR}$ results in wave propagation.

It has been suggested that propagation of Ca^{2+} waves is mediated by Ca^{2+} uptake into a highly loaded SR and subsequent activation of the release mechanism from within the SR (Takamatsu & Wier, 1990; Fabiato, 1992; Lukyanenko *et al.* 1996). To test this hypothesis directly, we examined the ability of thapsigargin to inhibit propagation of Ca^{2+} release following focal caffeine applications in cells already preloaded by Ca^{2+} above normal levels. Figure 3*A* illustrates the effects of exposure of the cells to thapsigargin (10 μM for 1 min) on the caffeine-induced responses in the presence of 3 mM Ca^{2+} in the bathing solution. Rather than inhibiting wave propagation, thapsigargin actually enhanced it (Fig. 3*B*). Again, the peak amplitude of the caffeine-induced responses was without a change, indicating no significant alterations in the SR Ca^{2+} load (Fig. 3*C*). Based on the results of six experiments, the velocities of Ca^{2+} waves before and after application of thapsigargin were 43·0 ± 1·2 and 50·2 ± 1·7 μm s^{-1}, respectively ($P < 0·05$). The effects of thapsigargin on space-averaged caffeine-induced Ca^{2+} transients are shown in Fig. 3*D*. Again, inhibition of the Ca^{2+} uptake capacity of the SR is confirmed by the reduced amplitude of the caffeine-induced Ca^{2+} signal in the presence of the drug. Similar results were obtained in four other experiments.

Since the enhancement of Ca^{2+} wave propagation by thapsigargin could be also due to a potentiation of the release mechanism by thapsigargin, we examined the effect of thapsigargin on SR Ca^{2+} release channels incorporated into lipid bilayers (not shown). In four channels the open probability (P_o) measured before and after application of 10 μM thapsigargin to the cytosolic side of the channel was 0·21 ± 0·03 and 0·26 ± 0·04, respectively. This result is consistent with the lack of effect of thapsigargin (100 μM) on Ca^{2+} release in purified muscle vesicles (Kirby *et al.* 1992). Taken together, these results suggest that Ca^{2+} uptake from the approaching wave front is not required for wave propagation.

Effects of low caffeine concentrations

It has been demonstrated that elevation of luminal Ca^{2+} increases the sensitivity of the Ca^{2+} release channel to cytosolic Ca^{2+} (Györke & Györke, 1998). Such an increase in Ca^{2+} sensitivity of the Ca^{2+} release mechanism, expected as a result of enhanced Ca^{2+} accumulation in the SR, could represent another mechanism for Ca^{2+} wave propagation in Ca^{2+}-overloaded myocytes. To investigate whether the conditions for propagation of Ca^{2+} waves can be created 'artificially' by sensitizing the release channels to cytosolic Ca^{2+} (i.e. without increasing the SR Ca^{2+} load), we tested the effects of low concentrations of caffeine on the ability of local caffeine pulses to trigger propagating releases. Caffeine is a known potentiator of CICR, which is believed to act by enhancing the sensitivity of the Ca^{2+} release channel to cytosolic Ca^{2+} (Endo, 1977). Figure 4 illustrates the results of exposure of cells to 0·5 mM caffeine under the conditions of reduced ($[Ca^{2+}]_o = 1$ mM) or elevated ($[Ca^{2+}]_o = 3$ mM) SR Ca^{2+} loads employed beforehand. Caffeine was applied to the whole bath 20 s prior to delivery of the focal 10 mM

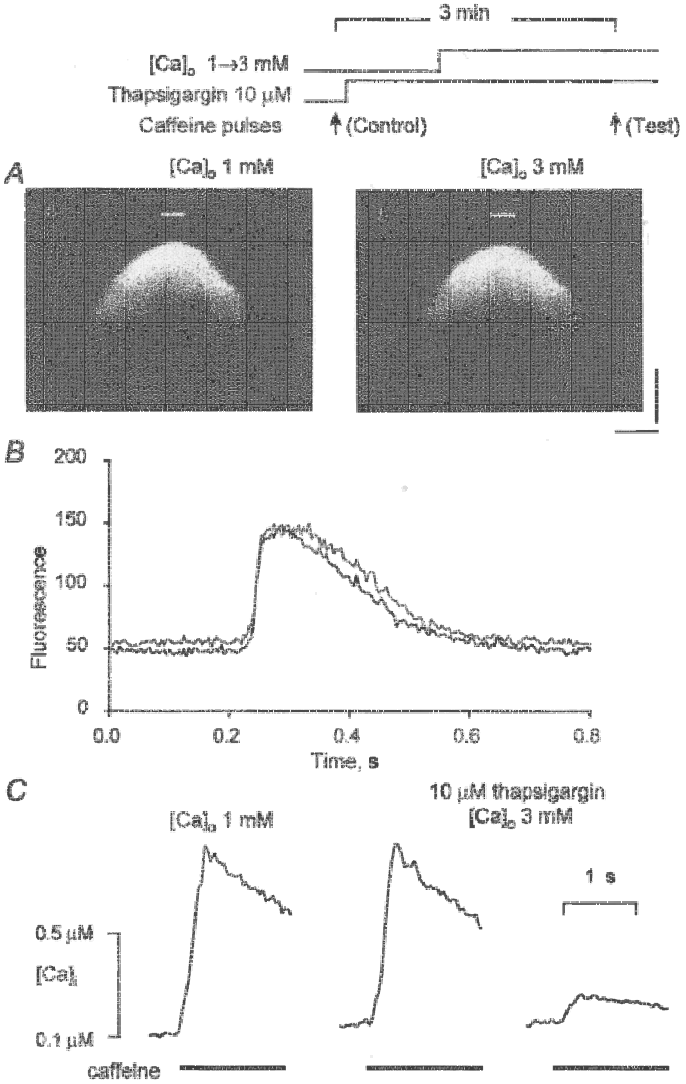

Figure 2. The effects of $[Ca^{2+}]_o$ on $[Ca^{2+}]_i$ responses to focal applications of caffeine in cells pretreated with thapsigargin

A, linescan images of fluorescence changes induced by focal applications of caffeine (10 mM) in the presence of 1 mM $[Ca^{2+}]_o$ (*a*) and 2 min after increasing $[Ca^{2+}]_o$ to 3 mM (*b*). Thapsigargin (10 μM) was introduced into the bath 1 min prior to elevating $[Ca^{2+}]_o$. The experimental protocol is presented schematically at the top. Calibration bars: horizontal, 18 μm; vertical, 0·64 s. *B*, time-dependent changes in fluorescence at the site of caffeine applications (marked in *Aa* and *b* by bars) at 1 mM $[Ca^{2+}]_o$ (black trace) and 3 mM $[Ca^{2+}]_o$ (grey trace). *C*, caffeine-induced Ca^{2+} transients measured before and after successive applications of thapsigargin and 3 mM Ca^{2+} to the bath. Caffeine (20 mM) was applied for 2 s. The lefthand trace is the control ($[Ca^{2+}]_o = 1$ mM). The middle trace was recorded 3 and 2 min after introduction to the bath of thapsigargin (10 μM) and elevated Ca^{2+} (3 mM), respectively. The righthand trace was acquired 2 min after the middle trace in the continuous presence of thapsigargin and 3mM Ca^{2+}.

caffeine pulse. At this combination of concentration and exposure time, caffeine used for conditioning of the release channels could effectively reach the channels inside the cell but at the same time did not cause premature Ca^{2+} release on a massive scale. It can be seen that with 1 mM $[Ca^{2+}]_o$ the 10 mM caffeine pulse again induced only an abortive Ca^{2+} response, which died out within 30 μm of the site of caffeine application (Fig. 4*Aa*). However, following pre-exposure of the cell to 0·5 mM caffeine the release became regenerative and propagated through the entire cell (Fig. 4*Ab*). This was despite the fact that the SR Ca^{2+} content might have been slightly reduced in the presence of 0·5 mM caffeine, as suggested by the decreased amplitude of the local caffeine-induced $[Ca^{2+}]$ transient (Fig. 4*C*). Interestingly, the velocity of the waves recorded under these conditions (i.e. 0·5 mM caffeine and 1 mM $[Ca^{2+}]_o$) was much lower than under our standard conditions with no caffeine and 3 mM $[Ca^{2+}]_o$ (31·4 ± 2·9 ($n = 12$) *vs.* 46·0 ± 2·3 ($n = 25$), $P < 0·05$; Fig. 4*B*). Such low wave velocities at reduced Ca^{2+} loads evidently result from reduced diffusion of Ca^{2+} out of the

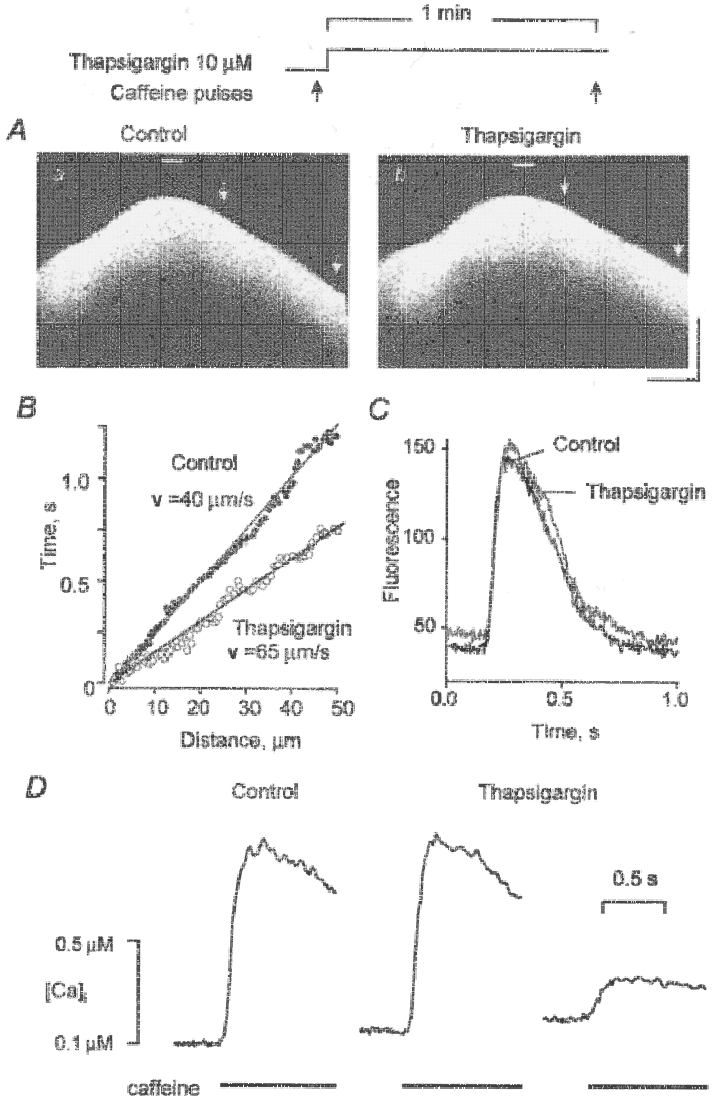

Figure 3. The effects of thapsigargin on $[Ca^{2+}]_i$ responses to focal applications of caffeine in cells preloaded with Ca^{2+} above normal levels

A, linescan images of fluorescence changes induced by focal applications of caffeine (10 mM) before (*a*) and 2 min after introducing of 10 μM thapsigargin to the bath (*b*). $[Ca^{2+}]_o$ was 3 mM. Calibration bars: horizontal, 18 μm; vertical, 0·66 s. *B*, linear fits to the positions of the wave in *Aa* (●) and *b* (○) in sections defined by the arrows; the values of mean wave velocities are indicated next to the respective linear fits. *C*, time-dependent changes in fluorescence at the sites of caffeine application (marked in *Aa* and *b* by bars) in the presence (black trace) and absence (grey trace) of thapsigargin. *D*, Ca^{2+} transients induced by applications of caffeine before (lefthand trace) and after introduction to the bath of 10 μM thapsigargin (middle and righthand traces). Caffeine (20 mM) was applied to the bath for 2 s at 2 min intervals; thapsigargin was applied 1 min prior to the second addition of caffeine.

wavefront due to a smaller wave amplitude (Stern, 1992). With 3 mM $[Ca^{2+}]_o$, propagating Ca^{2+} waves could be triggered under control conditions, but preconditioning with 0·5 mM caffeine caused a significant increase in the velocity of the wave (51·0 ± 3·4 *vs.* 65·2 ± 3·8, $n = 9$, $P < 0·05$; Fig. 5*A* and *B*). Exposure to 0·5 mM caffeine for periods longer than 0·5–1 min resulted in a decrease in both propagation ability and magnitude of the Ca^{2+} responses below their respective control levels (not shown). These changes were evidently caused by a progressive loss of the SR Ca^{2+} content in the presence of caffeine.

To quantify the potentiatory effects of 0·5 mM caffeine on the release mechanism, we performed measurements from single cardiac SR Ca^{2+} release channels incorporated into lipid bilayers. Ca^{2+} release channels were recorded using symmetrical 300 mM $CsMeSO_3$ in the presence of 3 mM cytosolic MgATP with 2 μM and 1 mM Ca^{2+} at the cytosolic and luminal sides of the channel, respectively. Figure 6 shows examples of traces acquired from a single channel before and after the addition of 0·5 mM caffeine to the cytosolic side of the channel. It can be seen that caffeine caused a dramatic potentiation of channel activity. On average, 0·5 mM caffeine increased the channel P_o measured for 1 min after addition of the drug by 400% (0·014 ± 0·003 *vs.* 0·056 ± 0·006, $n = 5$, $P < 0·05$). For comparison, increasing luminal Ca^{2+} from 20 μM to 2 mM under similar ionic conditions has been shown to increase P_o by 700% (Lukyanenko *et al.* 1996). Therefore, using 0·5 mM caffeine can indeed be regarded as a reasonable approximation of the effects of increased luminal Ca^{2+} on Ca^{2+} release channel activity.

Effects of tetracaine

To probe further the role of luminal Ca^{2+} in Ca^{2+} wave propagation, we examined the effects of the allosteric inhibitor of the RyR channel, tetracaine, on responses induced by focal caffeine applications. It has been shown that submaximal tetracaine concentrations have only a transient suppressing effect on spontaneous (i.e. Ca^{2+} sparks and waves) and electrically evoked Ca^{2+} release (Györke *et al.* 1997; Overend *et al.* 1997, 1998). The temporary nature of tetracaine inhibition has been attributed to the increased SR Ca^{2+} accumulation caused by the drug (presumably via inhibition of Ca^{2+} leakage through the Ca^{2+} release channels).

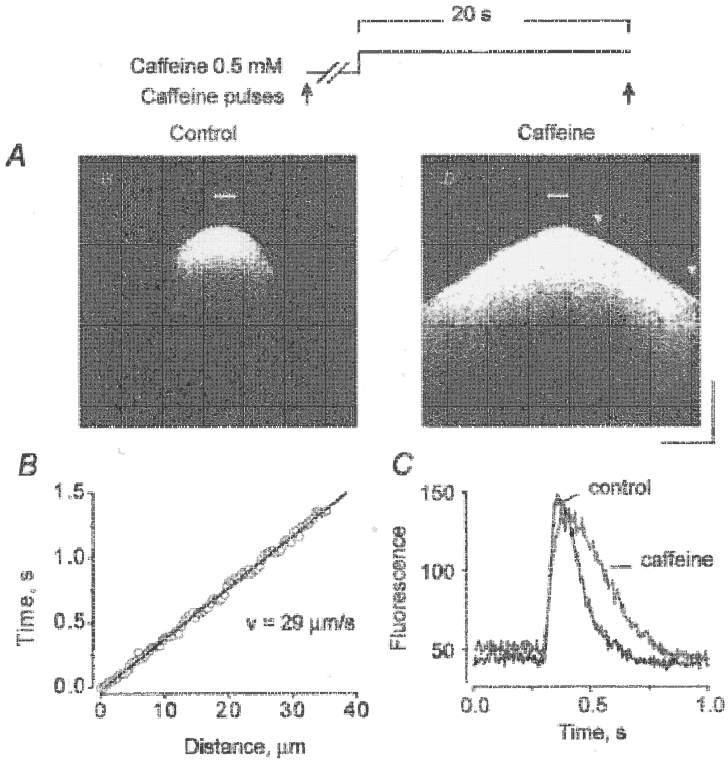

Figure 4. The effects of sensitizing the release channels to cytosolic Ca^{2+} by low doses of caffeine on the ability of local releases to induce propagating Ca^{2+} waves under conditions of reduced SR Ca^{2+} load

A, linescan images of fluorescence changes induced by focal applications of caffeine (10 mM) before (*a*) and 20 s after introducing 0·5 mM caffeine to the bath (*b*). The experimental protocol is illustrated by the scheme at the top. $[Ca^{2+}]_o$ was 1 mM. Calibration bars: horizontal, 18 μm; vertical, 0·66 s. *B*, linear fits to the position of the propagating Ca^{2+} wave for the section in *Ab* defined by the arrows; the value of the mean wave velocity (v) is indicated. *C*, time-dependent changes in fluorescence at the sites of caffeine application (marked in corresponding images in *A* by bars) with (black traces) and without (grey traces) conditioning by 0·5 mM caffeine.

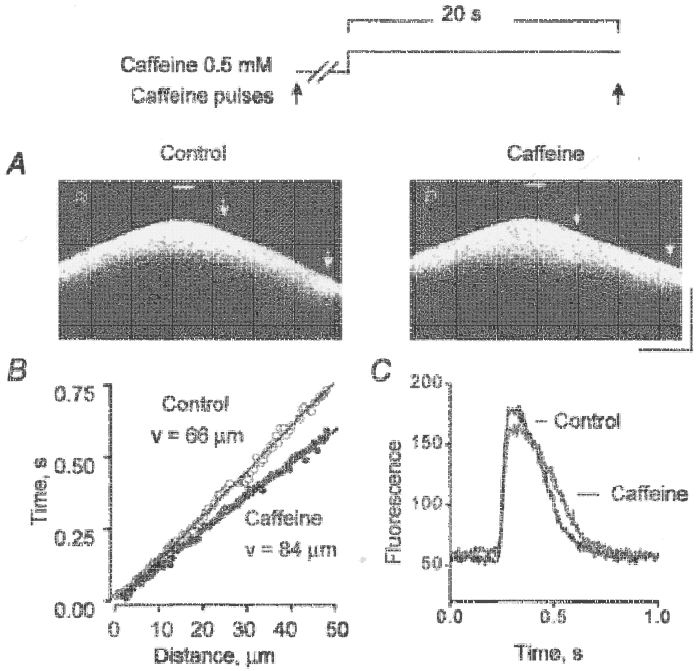

Figure 5. The effects of low doses of caffeine on Ca^{2+} wave propagation at increased SR Ca^{2+} load

A, linescan images of fluorescence changes induced by focal applications of caffeine (10mM) before (*a*) and 20 s after introducing 0·5 mM caffeine to the bath (*b*), as illustrated by the scheme at the top. $[Ca^{2+}]_o$ was 3 mM. Calibration bars: horizontal, 18 μm; vertical, 0·66 s. *B*, linear fits to the positions of the Ca^{2+} waves for sections defined by arrows in corresponding images; the values of mean wave velocities are indicated. *C*, time-dependent changes in fluorescence at the sites of caffeine applications (marked in the corresponding images by bars) with (black traces) and without (grey traces) conditioning by 0·5mM caffeine.

One possibility is that the recovery of release in the presence of tetracaine is due to the increased amount of releasable Ca^{2+} (Györke *et al.* 1997; Overend *et al.* 1997). Such a compensation of blockage could only occur when the inhibition by the drug is partial and at least some release sites remain available for liberation of Ca^{2+}. Alternatively, luminal Ca^{2+} alters the tetracaine sensitivity of the Ca^{2+} release channels through an allosteric mechanism, making the channels more resistant to tetracaine (Györke *et al.* 1997). With such a removal of blockage, a recovery of release

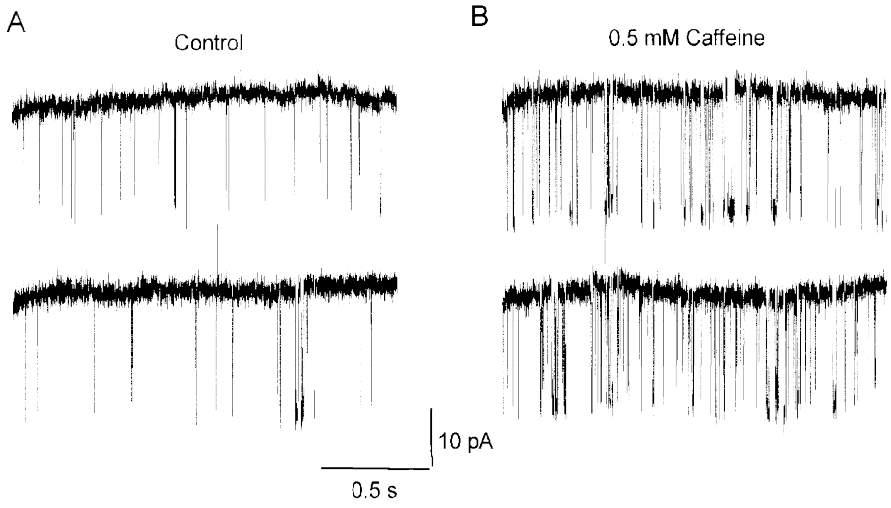

Figure 6. The effects of 0·5 mM caffeine on the activity of single cardiac RyR channels incorporated into lipid bilayers

A and *B*, representative single channel traces recorded before (*A*) and after (*B*) introduction of 0·5 mM caffeine into the *cis* chamber. Current recordings were obtained at −40mV holding potential in symmetrical 350 mM $CsCH_3SO_3$, pH 7·4. The *cis* chamber contained 3 mM MgATP and 2 μM free $[Ca^{2+}]$. The *trans* chamber contained 1 mM Ca^{2+}.

could occur even following a complete initial inhibition. The effects of tetracaine on caffeine-induced Ca^{2+} waves are summarized in Fig. 7. Exposure of the cells to 0·75 mM tetracaine for 1 min resulted in a complete inhibition of caffeine-induced Ca^{2+} release of all forms (Fig. 7*Aa* and *b*). However, further continuous incubation of the cells with the drug resulted in re-emergence of first localized (1 min, Fig. 7*Ac*) and then propagating Ca^{2+} releases (3 min, Fig. 7*Ad*). The amplitude of the fluorescence signal at the site of caffeine application was significantly increased compared with the reference situation (Fig. 7*B*). Similar results were obtained in eight other experiments. This is consistent with an increase in the SR Ca^{2+} content caused by tetracaine (Györke *et al.* 1997; Overend *et al.* 1997). The increased accumulation of Ca^{2+} in the SR in the presence of tetracaine was confirmed in experiments with application of caffeine to the entire cell (Fig. 7*C*). The magnitude of the caffeine-induced Ca^{2+} transients increased by 39 % ± 7 % ($n = 4$) after 3 min of incubation with 0·75 mM tetracaine. Based on these results we conclude that the recovery of release from inhibition in the presence of tetracaine is not simply due to a compensation of blockage by increased SR Ca^{2+} load. Instead, the release mechanism itself becomes altered in a way that makes it less sensitive to inhibition by tetracaine. These results further support the possibility that a modulation of the release mechanism by luminal Ca^{2+} may be involved in the generation of Ca^{2+} waves in cardiac myocytes.

DISCUSSION

Growing evidence suggests that the activity of Ca^{2+} release channels of the SR can be influenced by luminal Ca^{2+} (Fabiato, 1992; Sitsapesan & Williams, 1994; Bassani *et al.* 1995; Lukyanenko *et al.* 1996; Györke *et al.* 1997; Santana *et al.* 1997; Györke & Györke, 1998). The present study is the first systematic attempt to define the potential role of this mechanism in the generation and propagation of Ca^{2+} waves in cardiac myocytes under conditions of increased Ca^{2+} loading. Two specific hypotheses were considered: (1) Increased luminal Ca^{2+} sensitizes the Ca^{2+} release channels to cytosolic Ca^{2+}, enhancing the ability of cytosolic Ca^{2+} to activate adjacent release sites via CICR. (2) Ca^{2+} transported from the wavefront into the adjacent SR elements raises luminal Ca^{2+} above a critical threshold level, resulting in activation of the release channels at a luminal site.

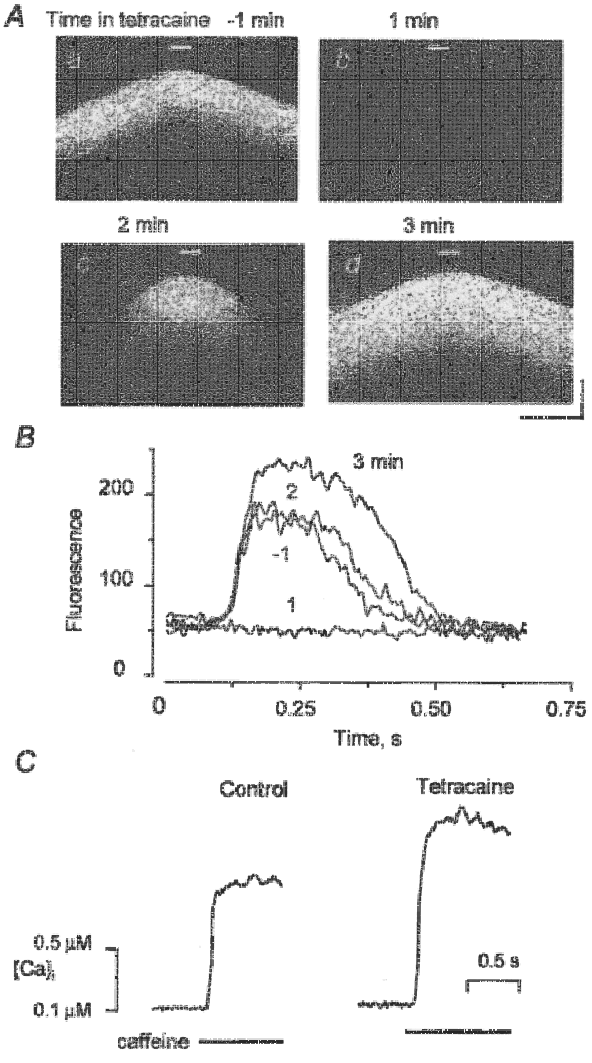

Figure 7. The effect of tetracaine on $[Ca]_i$ responses induced by focal caffeine applications

A, linescan images of fluorescence changes acquired under control conditions (*a*) and at different times (1, 2 and 3 min; *b*, *c* and *d*, respectively) after addition to the bath of 0·75 mM tetracaine. $[Ca^{2+}]_o$ was 3 mM. Calibration bars: horizontal, 10 μm; vertical, 0·33 s. *B*, time-dependent changes in fluorescence at the site of caffeine applications (marked by bars) for the images in *A*, as indicated. *C*, Ca^{2+} transients elicited by application of 20 mM caffeine to the whole bath before (lefthand trace) and 3 min after exposure of the cell to 0·75 mM tetracaine (righthand trace).

To trigger and measure propagating Ca^{2+} waves we used focal caffeine application in combination with linescan confocal Ca^{2+} imaging. We created conditions for the transition of localized Ca^{2+} releases into propagating Ca^{2+} waves by increasing the cellular Ca^{2+} load via elevation of extracellular $[Ca^{2+}]$. This procedure is known to increase $[Ca^{2+}]$ in both cytosolic and SR luminal compartments of cardiac myocytes (Wier *et al.* 1987; Lukyanenko *et al.* 1996; and the present study). We found that selectively inhibiting the accumulation of extra Ca^{2+} into the luminal compartment by thapsigargin prevented the transition of non-propagating Ca^{2+} responses to propagating Ca^{2+} waves upon elevation of $[Ca^{2+}]_o$. This result provides a direct experimental confirmation of the common assumption that increased SR Ca^{2+} content plays a critical role in the generation of propagating Ca^{2+} waves in Ca^{2+}-overloaded cardiac myocytes. We further found that inhibition of the SR Ca^{2+} uptake into the SR with thapsigargin did not prevent Ca^{2+} wave propagation in cells already preloaded with Ca^{2+} above normal levels. Moreover, the velocity of the waves was accelerated relative to the control conditions (Fig. 3). This result shows unequivocally that propagation of Ca^{2+} waves does not require Ca^{2+} uptake from the approaching wave front. This conclusion is consistent with the relatively weak temperature dependency of Ca^{2+} wave propagation (Engel *et al.* 1995) and the rapid kinetics of local $[Ca^{2+}]$ rise during the wave (Cheng *et al.* 1996) shown recently in rat ventricular myocytes.

Another important finding of the present study was that sensitizing the release channels to cytosolic Ca^{2+} by low concentrations of caffeine dramatically augmented the ability of local releases to induce propagating Ca^{2+} waves. These results showed for the first time that propagating Ca^{2+} waves could be produced solely by enhancing the activity of the Ca^{2+} release channels without increasing the cellular Ca^{2+} load. These results are consistent with the hypothesis that potentiation of the Ca^{2+} release channel activity by elevated luminal Ca^{2+} may be involved in propagation of Ca^{2+} waves in cardiac myocytes. This hypothesis was further supported by the results of our experiments with the allosteric blocker of the RyR channel, tetracaine. We have recently demonstrated in lipid bilayer experiments that increased luminal Ca^{2+} modifies the RyRs in a fashion that makes them more sensitive to activation by cytosolic Ca^{2+} and more resistant to inhibition by cytosolic Ca^{2+} and tetracaine (Györke *et al.* 1997; Györke & Györke, 1998). In the present study we used a tetracaine resistance test to confirm that RyRs also become allosterically altered in intact myocytes upon increasing the SR Ca^{2+} load. We showed that tetracaine (0·75 mM) initially inhibited fully both localized and propagating responses triggered by focal caffeine applications. This inhibition was, however, completely reversed by the increased accumulation of Ca^{2+} in the SR caused by the drug (Fig. 7). We speculate that the tetracaine-resistant conformation with altered Ca^{2+} gating is the form of the channel that is involved in Ca^{2+} wave generation in cardiac myocytes.

To better understand how alterations in the activity of the release channels could affect the generation of Ca^{2+} waves, we performed computer simulations using a numerical model of the Ca^{2+} wave. Recently, Keizer *et al.* (1998) showed that a simple 'fire–diffuse–fire' model can account for saltatory wave propagation in cardiac myocytes. In their work, they numerically solved a single reaction diffusion model for free $[Ca^{2+}]$ in one spatial dimension. We expanded upon the Keizer model by including the effects of soluble Ca^{2+} buffers, Ca^{2+}:dye complex and the free dye. Immunochemical and ultrastructural evidence indicate that SR Ca^{2+} release channels are localized in junctional and corbular SR, which occur at the level of Z-lines and t-tubules (Jorgensen *et al.* 1993; Franzini-Armstrong & Protasi, 1997). In addition, elementary Ca^{2+} release events – Ca^{2+} sparks – have been shown to originate at t-tubules in cardiac cells (Shacklock *et al.* 1995; Cheng *et al.* 1996). Therefore, we mimicked the subsarcomeric localization of the Ca^{2+} release channels by placing the release sites at a distance of 2 μm from each other. The parameters of release, diffusion, buffering and uptake were selected from the accepted range of literature values so as to maximize uninterrupted diffusion of Ca^{2+} between the release sites (Table 1). Nuclear magnetic resonance studies have shown that upon increasing the cellular Ca^{2+} load, luminal $[Ca^{2+}]$ can rise up to 5 mM (Chen *et al.* 1996). Thus to mimic the rise of luminal $[Ca^{2+}]$ under conditions of increased cellular Ca^{2+} loads, $[Ca^{2+}]_{SR}$ was set at 5 mM. Using this model, we investigated the range for the Ca^{2+} threshold of release site activation required for successful wave propagation. Ca^{2+} waves were initiated by triggering Ca^{2+} release in a region with a diameter of 6 μm containing three release sites. Examples of theoretical linescan responses generated by using four different threshold values (5, 2, 1·3 and 0·5 μM) are presented in Fig. 8. In accordance with simulations by Keizer and co-authors (1998) the propagating Ca^{2+} waves exhibited clear saltatory properties. With the threshold set at 5 μM, the local Ca^{2+} elevation did not initiate Ca^{2+} release from the release sites (Fig. 8*a*). With a threshold of 2 μM the same stimulus triggered CICR that propagated to both sides by sequential activation of release sites for a distance of about 10 μm but then died out spontaneously (Fig. 8*b*). When the Ca^{2+} threshold was set below 1·5 μM the same local Ca^{2+} elevations triggered self-sustaining Ca^{2+} waves which propagated over the entire domain of simulation (Fig. 8*c* and *d*). The velocity of the wave was higher for simulations that employed lower Ca^{2+} activation threshold settings (cf. Fig. 8*d* and *c*). The results of these simulations are in accordance with our experimental results, which showed that sensitizing the release mechanism to cytosolic Ca^{2+} enhanced wave generation and propagation in cardiac myocytes (Fig. 5). Our experimental images of Ca^{2+} waves did not show the clear site-to-site propagation seen in the images of theoretical Ca^{2+} waves. This apparent discrepancy could be ascribed to the limited spatial and temporal resolution provided by the confocal microscope. A similar problem in detecting localized release events during both

normal E–C coupling and Ca^{2+} waves has been described previously (Cheng *et al.* 1993, 1996; Cannel *et al.* 1994). It has been shown that image enhancement analysis can reveal Ca^{2+} wave propagation by sequential activation of release sites (Cheng *et al.* 1996).

It is interesting to note that in Fig. 8*b*, the wave propagated only 10 μm when the threshold was 2 μM. The reason for termination is that there is a net depletion of calcium in the wavefront, i.e. $q_{rel} - q_{rem} < 0$. The wave will terminate when the local free calcium concentration falls below the firing threshold. Increasing the magnitude of the initial trigger signal while holding the firing threshold fixed resulted in increased wave propagation length (not shown). Our model therefore predicts that an abortive (i.e. intrinsically not self-sustaining) wave propagates only over the region in which the initial calcium release has a diffusive influence.

Our computer simulations indicate that even with fairly large Ca^{2+} releases and with parameters of the model favouring Ca^{2+} diffusion, a localized release fails to initiate a self-sustaining Ca^{2+} wave for thresholds $\geqslant 1{\cdot}3$ μM. In rat ventricular myocytes under normal SR Ca^{2+} loading conditions, the cytosolic Ca^{2+} concentration required for activation of a Ca^{2+} spark has been estimated to be close to 15 μM (Cannel *et al.* 1994). This value is consistent with the low Ca^{2+} sensitivity of the RyR channels ($K_D \approx 30$ μM; Györke & Györke, 1998) in reconstitution experiments in the presence of physiological concentrations of Mg^{2+} and ATP. Studies from our laboratory have shown that increased luminal $[Ca^{2+}]$ enhances the sensitivity of the RyRs to cytosolic $[Ca^{2+}]$ (Lukyanenko *et al.* 1996; Györke & Györke, 1998). In particular, elevating luminal Ca^{2+} from 0·2–1 to 5–10 mM induced a 2- to 7-fold increase in the P_o of RyRs activated by cytosolic $[Ca^{2+}]$ (Györke & Györke, 1998). Values for resting free $[Ca^{2+}]_{SR}$ have been estimated to be in the 0·7–1·5 mM range at loading conditions that can be considered normal (Chen *et al.* 1996; Shannon & Bers, 1997). Under conditions of increased cellular Ca^{2+} load, free $[Ca^{2+}]_{SR}$ can rise to 5 mM (Chen *et al.* 1996). Such an elevation in $[Ca^{2+}]_{SR}$ would be expected to augment RyR channel activity, bringing the threshold of activation of the RyRs by cytosolic Ca^{2+} closer to the range that is required for Ca^{2+} wave propagation according to our simulations. Therefore, the results of our Ca^{2+} wave simulations suggest that increased amounts of releasable Ca^{2+} alone may not be sufficient to account for the transition of local releases into propagating Ca^{2+} waves under conditions of increased SR Ca^{2+} loads. However, sensitization of the release sites to activating Ca^{2+} can effectively promote the generation of self-sustaining Ca^{2+} waves.

Taken together, the results of the present study suggest that the potentiation of SR Ca^{2+} release channels by luminal Ca^{2+} is an important factor in the generation of self-sustaining Ca^{2+} waves in rat ventricular myocytes. Ca^{2+} wave propagation does not require the translocation of Ca^{2+} from

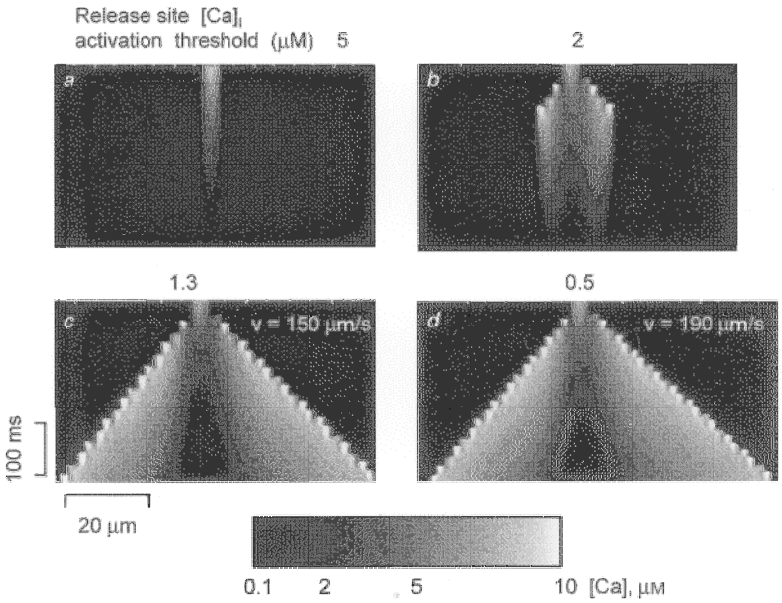

Figure 8. The dependence of theoretical $[Ca^{2+}]_i$ responses on the threshold of release site Ca^{2+} activation

a–d, simulated linescan images of $[Ca^{2+}]_i$ responses at different Ca^{2+} activation thresholds (5, 2, 1·3 and 0·5 μM, respectively). The $[Ca^{2+}]_i$ responses were triggered by a Ca^{2+} elevation produced by an exponentially decaying release flux with a peak amplitude of 30 mM s^{-1} and decay time constant of 2 ms in a region with a diameter of 6 μm. The simulation included 50 individual release sites with a diameter of 0·1 μm each spaced at intervals of 2 μm, 2 spatially homogeneous removal fluxes (SR Ca^{2+} pumps and soluble buffers), diffusible dye and the Ca^{2+}:dye complex. The values of the mean wave velocities (v, μm s^{-1}) in *c* and *d* are indicated.

the spreading wave front into the SR. Instead it appears to rely on the steadier effects of luminal Ca^{2+}, consisting of sensitization of the Ca^{2+} release channels to cytosolic Ca^{2+}. These results may have implications for understanding mechanisms of Ca^{2+} waves in other cell types where modulatory effects of luminal Ca^{2+} on the release mechanism have been implicated (Nunn & Taylor, 1992).

Albritton, N. L., Meyer, T. & Stryer, L. (1992). Range of messenger action of calcium ion and inositol 1,4,5-trisphosphate. *Science* **258**, 1812–1815.

Bassani, J. W., 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., Yuan, W. & Bers, D. M. (1995). Fractional SR Ca^{2+} release is regulated by trigger Ca^{2+} and SR Ca^{2+} content in cardiac myocytes. *American Journal of Physiology* **268**, C1313–1329.

Bers, D. M. (1991). *Excitation–Contraction Coupling and Cardiac Contractile Force.* Kluwer Academic Publishers, Dordrecht, The Netherlands.

Beuckelmann, D. J. & Wier, W. G. (1988). Mechanism of release of calcium from sarcoplasmic reticulum of guinea-pig cardiac cells. *Journal of Physiology* **405**, 233–255.

Cannell, M. B., Berlin, J. R. & Lederer, W. J. (1987). Effect of membrane potential changes on the calcium transient in single rat cardiac muscle cells. *Science* **238**, 1419–1423.

Cannell M. B., Cheng H. & Lederer W. J. (1994). Spatial non-uniformities in $[Ca^{2+}]_i$ during excitation–contraction coupling in cardiac myocytes. *Biophysical Journal* **67**, 1942–1956.

Chen, W., Steenbergen, C., Levy, L., Vance, J. & Murphy, E. (1996). Measurement of free Ca^{2+} in sarcoplasmic reticulum in perfused rabbit heart loaded with 1,2-bis(2-amino-5,6-difluorophenoxy)ethane-*N*,*N*,*N*′-tetraacetic acid by ^{19}F NMR. *Journal of Biological Chemistry* **29**, 7398–7403.

Cheng, H., Lederer, W. & Cannell, M. B. (1993). Calcium sparks: elementary events underlying excitation–contraction coupling in heart muscle. *Science* **262**, 740–744.

Cheng, H., Lederer, M. R., Lederer, W. J. & Cannell, M. B. (1996). Calcium sparks and $[Ca^{2+}]_i$ waves in cardiac myocytes. *American Journal of Physiology* **270**, C148–159.

Dettbarn, C., Györke, S. & Palade, P. (1994). Many agonists induce 'quantal' Ca^{2+} release or adaptive behavior in muscle ryanodine receptors. *Molecular Pharmacology* **46**, 502–507.

Eisner, D. A., Trafford, A. W., Diaz, M. E., Overend, C. L. & O'Neill, S. C. (1998). The control of Ca release from the cardiac sarcoplasmic reticulum: regulation *versus* autoregulation. *Cardiovascular Research* **38**, 589–604.

Endo, M. (1977). Calcium release from the sarcoplasmic reticulum. *Physiological Reviews* **57**, 71–108.

Engel, J., Sowerby, A. J., Finch, A. E., Fechner, M. & Stier, A. (1995). Temperature dependence of Ca^{2+} wave properties in cardiomyocytes: implications for the mechanism of autocatalytic Ca^{2+} release in wave propagation. *Biophysical Journal* **68**, 40–45.

Escobar, A. L., Cifuentes, F. & Vergara, J. L. (1995). Detection of Ca^{2+}-transients elicited by flash photolysis of DM-nitrophen with a fast calcium indicator. *FEBS Letters* **364**, 335–338.

Fabiato, A. (1983). Calcium-induced release of calcium from the cardiac sarcoplasmic reticulum. *American Journal of Physiology* **245**, C1–14.

Fabiato, A. (1992). Two kinds of calcium-induced release of calcium from the sarcoplasmic reticulum of skinned cardiac cells. In: *Excitation–Contraction Coupling in Skeletal, Cardiac and Smooth Muscle*, ed. Frank, G. B., pp. 245–262. Plenum Press, New York.

Franzini-Armstrong, C. & Protasi, F. (1997). Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiological Reviews* **77**, 699–729.

Györke, I. & Györke, S. (1998). Regulation of the cardiac ryanodine receptor channel by luminal Ca involves luminal Ca sensing sites. *Biophysical Journal* (in the Press).

Györke, S., Lukyanenko, V. & Györke, I. (1997). Dual effects of tetracaine on spontaneous calcium release in rat ventricular myocytes. *Journal of Physiology* **500**, 297–309.

Györke, S. & Palade, P. (1993). Role of local Ca^{2+} domains in activation of Ca^{2+}-induced Ca^{2+} release in crayfish muscle. *American Journal of Physiology* **264**, C1505–1512.

Han, S., Schiefer, A. & Isenberg, G. (1994). Ca^{2+} load of guinea-pig ventricular myocytes determines efficacy of brief Ca^{2+} currents as trigger for Ca^{2+} release. *Journal of Physiology* **480**, 411–421.

Harkins, A. B., Kurebayashi, N. & Baylor, S. M. (1993). Resting myoplasmic free calcium in frog skeletal muscle fibers estimated with fluo-3. *Biophysical Journal* **65**, 865–881.

Ishide, N. (1996). Intracellular calcium modulators for cardiac muscle in pathological conditions. *Japanese Heart Journal* **37**, 1–17.

Janczewski, A. M. J., Spurgeon, H. A., Stern, M. D. & Lakatta, E. G. (1994). Effects of sarcoplasmic reticulum Ca^{2+} load on the gain function of Ca^{2+} release by Ca^{2+} current in cardiac cells. *American Journal of Physiology* **268**, H916–920.

Jorgensen, A. O., Shen, A. C., Arnold, W., McPherson, P. S. & Campbell, K. P. (1993). The Ca^{2+} release channel/ryanodine receptor is localized in junctional corbular sarcoplasmic reticulum in cardiac muscle. *Journal of Cell Biology* **120**, 969–980.

Keizer, J., Smith, G., Ponce-Dawson, S. & Pearson, J. (1998). Saltatory propagation of Ca^{2+} waves by Ca^{2+} sparks. *Biophysical Journal* **75**, 595–600.

Kirby, M. S., Sagara, Y., Gaa, S., Inesi, G., Lederer, W. J. & Rogers, T. B. (1992). Thapsigargin inhibits contraction and Ca^{2+} transient in cardiac cells by specific inhibition of the sarcoplasmic reticulum Ca^{2+} pump. *Journal of Biological Chemistry* **25**, 12545–12551.

Kort, A., Capogrossi, M. & Lakatta, E. (1985). Frequency, amplitude, and propagation velocity of spontaneous Ca^{2+}-dependent contractile waves in intact adult rat cardiac muscle and isolated myocytes. *Circulation Research* **57**, 844–855.

Lipp, P. & Niggli, E. (1994). Modulation of Ca^{2+} release in cultured neonatal rat cardiac myocytes. *Circulation Research* **74**, 979–990.

Lopez-Lopez, 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.

Lukyanenko, V., Györke, I. & Györke, S. (1996). Regulation of calcium release by calcium inside the sarcoplasmic reticulum in ventricular myocytes. *Pflügers Archiv* **432**, 1047–1054.

Lukyanenko, V., Wiesner, T. & Györke, S. (1998). Inactivation of Ca^{2+} release during Ca^{2+} sparks in rat ventricular myocytes. *Journal of Physiology* **507**, 667–677.

Niggli, E. & Lederer, W. J. (1990). Voltage-independent calcium release in heart muscle. *Science* **250**, 565–568.

Nunn, D. L. & Taylor, C. W. (1992). Luminal Ca^{2+} increases the sensitivity of Ca^{2+} stores to inosytol 1,4,5-trisphosphate. *Molecular Pharmacology* **41**, 115–119.

Overend, C. L., Eisner, D. A. & O'Neill, C. O. (1997). The effect of tetracaine on spontaneous Ca^{2+} release and sarcoplasmic reticulum calcium content in rat ventricular myocytes. *Journal of Physiology* **502**, 471–479.

Overend, C. L., O'Neill, C. O. & Eisner, D. A. (1998). The effect of tetracaine on stimulated contractions, sarcoplasmic reticulum Ca^{2+} content and membrane current in isolated rat ventricular myocytes. *Journal of Physiology* **507**, 759–769.

Santana, L. F., Gomez, A. M., Kranias, E. G. & Lederer, W. J. (1997). Amount of calcium in the sarcoplasmic reticulum: influence on excitation–contraction coupling in heart muscle. *Heart Vessels* **12**, suppl., 44–49.

Satoh, H., Blatter, L. A. & Bers, D. M. (1997). Effects of $[Ca^{2+}]_i$, SR Ca^{2+} load, and rest on Ca^{2+} spark frequency in ventricular myocytes. *American Journal of Physiology* **272**, H657–668.

Shacklock, P. S., Wier, W. G. & Balke, C. W. (1995). Local Ca^{2+} transients (Ca^{2+} sparks) originate at transverse tubules in rat heart cells. *Journal of Physiology* **487**, 601–608.

Shannon, T. R. & Bers, D. M. (1997). Assessment of intra-SR free [Ca] and buffering in rat heart. *Biophysical Journal* **73**, 1524–1531.

Sipido, K. R. & Wier, W. G. (1991). Flux of Ca^{2+} across the sarcoplasmic reticulum of guinea-pig cardiac cells during excitation–contraction coupling. *Journal of Physiology* **435**, 605–630.

Sitsapesan, R. & Williams, A. J. (1994). Regulation of the gating of the sheep cardiac sarcoplasmic reticulum Ca^{2+}-release channel by luminal Ca^{2+}. *Journal of Membrane Biology* **137**, 215–226.

Smith, G. D., Keizer, J. E., Stern, M. D., Lederer, W. J. & Cheng, H. A. (1998). Simple numerical model of calcium spark formation and detection in cardiac myocytes. *Biophysical Journal* **75**, 15–32.

Song, L. S., Stern, M. D., Lakatta, E. G. & Cheng, H. (1997). Partial depletion of sarcoplasmic reticulum calcium does not prevent calcium sparks in rat ventricular myocytes. *Journal of Physiology* **505**, 665–675.

Stern, M. D. (1992). Theory of excitation–contraction coupling in cardiac muscle. *Biophysical Journal* **63**, 495–517.

Stern, M. D., Capogrossi, M. C. & Lakatta, E. G. (1988). Spontaneous calcium release from the sarcoplasmic reticulum in myocardial cells: mechanisms and consequences. *Cell Calcium* **9**, 247–256.

Stern, M. D. & Lakatta, E. (1992). Excitation–contraction in the heart: the state of the question. *FASEB Journal* **6**, 3092–3100.

Takamatsu, T. & Wier, W. G. (1990). Calcium waves in mammalian heart: quantification of origin, magnitude, waveform, and velocity. *FASEB Journal* **4**, 1519–1525.

Tang, Y. & Othmer, H. G. (1994). A model of calcium dynamics in cardiac myocytes based on the kinetics of ryanodine-sensitive calcium channels. *Biophysical Journal* **67**, 2223–2235.

Thastrup, O., Dawson, A. P., Scharff, O., Foder, B., Cullen, P. J., Drobak, B. K., Bjerrum, P. J., Christensen, S. B. & Hanley, M. R. (1989). Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. *Agents Actions* **27**, 17–23.

Trafford, A. W., Lipp, P., O'Neil, C. O., Niggli, E. & Eisner, D. A. (1995). Propagating calcium waves initiated by local caffeine application in rat ventricular myocytes. *Journal of Physiology* **489**, 319–326.

Trafford, A. W., O'Neill, S. C. & Eisner, D. A. (1990). Factors affecting the propagation of locally activated systolic Ca transients in rat ventricular myocytes. *Pflügers Archiv* **425**, 181–183.

Wier, W. G., Cannell, M. B., Berlin, J. R., Marban, E. & Lederer, W. J. (1987). Cellular and subcellular heterogeneity of $[Ca^{2+}]_i$ in single heart cells revealed by Fura-2. *Science* **235**, 325–328.

Wussling, M. H. P. & Salz, H. (1996). Nonlinear propagation of spherical calcium waves in rat cardiac myocytes. *Biophysical Journal* **70**, 1144–1153.

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