Dual effects of tetracaine on spontaneous calcium release in rat ventricular myocytes

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1. Confocal microfluorometry was used to study the effects of tetracaine on spontaneous Ca^{2+} release from the sarcoplasmic reticulum (SR) in isolated rat ventricular myocytes.

2. At low concentrations (0·25–1·25 mM), tetracaine caused an initial inhibition of spontaneous release events (Ca^{2+} sparks) and Ca^{2+} waves, which was followed by a gradual increase in Ca^{2+} release activity. The frequency and magnitude of sparks were first decreased and then increased with respect to control levels. At high concentrations (>1·25 mM), tetracaine abolished all forms of spontaneous release.

3. Exposure of the myocytes to tetracaine resulted in a gradual increase in the SR Ca^{2+} load as indexed by changes in the magnitude of caffeine-induced Ca^{2+} transients.

4. In cardiac SR Ca^{2+}-release channels incorporated into lipid bilayers, tetracaine (>0·25 mM) induced a steady inhibition of channel activity. Addition of millimolar Ca^{2+} to the luminal side of the channel caused an increase in channel open probability under control conditions as well as in the presence of various concentrations of tetracaine.

5. We conclude that the primary effect of tetracaine on SR Ca^{2+}-release channels is inhibition of channel activity both *in vitro* and *in situ*. The ability of tetracaine to reduce spark magnitude suggests that these events are not due to activation of single channels or non-reducible clusters of channels and, therefore, supports the multichannel origin of sparks. We propose that the paradoxical late potentiation of release by submaximal concentrations of tetracaine is caused by a gradual increase in SR Ca^{2+} load and subsequent activation of the Ca^{2+}-release channels by Ca^{2+} inside the SR.

In mammalian ventricular myocytes the process of excitation–contraction (E–C) coupling is mediated by Ca^{2+} influx from the extracellular milieu triggering Ca^{2+}-induced Ca^{2+} release (CICR) from the sarcoplasmic reticulum (SR) (Stern & Lakatta, 1992; Niggli & Lipp, 1995). Under certain conditions, when the Ca^{2+} content of the cell becomes sufficiently high (Ca^{2+} overload), myocytes exhibit another form of Ca^{2+} release that starts spontaneously in a small area and then propagates along the cell as a regenerative Ca^{2+} wave (Wier, Cannell, Berlin, Marban & Lederer, 1987; Lipp & Niggli, 1994; Trafford, Lipp, O'Neil, Niggli & Eisner, 1995; Engel, Sowerby, Finch, Fechner & Stier, 1995; Cheng, Lederer, Lederer & Cannell, 1996). Despite years of effort, the mechanisms of initiation of spontaneous Ca^{2+} release and their relation to Ca^{2+} release during normal E–C coupling are not precisely understood. Spontaneous Ca^{2+} release could be mediated by the normal process of CICR, which involves Ca^{2+} acting at a site on the cytoplasmic side of the release channel. In particular, spontaneous release could be initiated, as a result of a critical combination of SR and cytoplasmic Ca^{2+}, whenever the gain of the positive feedback loop inherent in CICR exceeds unity (Stern, Capogrossi & Lakatta, 1988; Stern, 1992; Cheng *et al.* 1996). Alternatively, spontaneous Ca^{2+} release could be triggered by high concentrations of Ca^{2+} acting at sites inside the SR (Fabiato, 1992; Bassani, Yuan & Bers, 1995; Lukyanenko, Györke & Györke, 1996).

Recently, it has been demonstrated that spontaneous Ca^{2+} release occurs normally in quiescent cells in the form of local spontaneous release events or Ca^{2+} sparks (Cheng, Lederer & Cannel, 1993; Lipp & Niggli, 1994). The Ca^{2+} spark is a transient and highly localized elevation of $[Ca^{2+}]$ that is believed to result from spontaneous openings of one or a few release channels (Cheng *et al.* 1993); however, it may also represent a regenerative cluster of a rather large number of channels (Lipp & Niggli, 1996). Under conditions of Ca^{2+} overload, both the frequency and magnitude of the sparks increase and these local Ca^{2+} elevations apparently become initiation sites of regenerative Ca^{2+} waves (Cheng *et al.* 1993, 1996; Lukyanenko *et al.* 1996).

Tetracaine and other local anaesthetics have been used extensively as research tools for studying E–C coupling in both skeletal and cardiac muscle. Studies in intact

(Chapman & Miller, 1974; Hunter, Haworth & Berkoff, 1982) and skinned cardiac myocytes (Stephenson & Wendt, 1986), isolated SR preparations (Chamberlain, Volpe & Fleischer, 1984; Meissner & Henderson, 1987; O'Brien, Valdivia & Block, 1995), as well as single SR Ca^{2+}-release channels (ryanodine receptors, RyRs) incorporated into lipid bilayers (Zahradnikova & Palade, 1993), have indicated that tetracaine and some other local anaesthetics inhibit the SR Ca^{2+}-release channels. Local anaesthetics (e.g. procaine), at least under certain conditions, can also potentiate caffeine-induced Ca^{2+} release in skinned cardiac cells, presumably through the increased accumulation of Ca^{2+} in the SR (Stephenson & Wendt, 1986). By virtue of reducing the number of active release channels (thus decreasing the feedback gain of CICR) but at the same time promoting Ca^{2+} accumulation in the SR, tetracaine may be a good experimental probe for evaluating the relative roles of a regenerative CICR and an intraluminal Ca^{2+}-activation mechanisms in the generation of spontaneous Ca^{2+} release. In this report we correlate the effects of tetracaine on spontaneous local release events and propagating Ca^{2+} waves, monitored by confocal Ca^{2+} imaging, with the effects of the drug on SR Ca^{2+}-release channels in lipid bilayers. Our results show that tetracaine inhibits the SR Ca^{2+} release channels both *in vitro* and *in situ*, but in cardiac myocytes, tetracaine also can lead to a 'paradoxical' potentiation of spontaneous Ca^{2+} release through an increase in SR Ca^{2+} load and subsequent activation of release channels by Ca^{2+} on the luminal side of the channel.

METHODS

Cell isolation and experimental solutions

Adult Sprague–Dawley rats (200–300 g) were killed by lethal injection of Nembutal (100 mg kg^{-1}, I.P.; Abbott Laboratories, Chicago, IL, USA). Single ventricular myocytes were obtained by enzymatic dissociation (Yasui, Palade & Györke, 1994). Briefly, Langendorff perfusion of the rat heart was carried out by using a Joklik minimum essential medium (37 °C, Sigma) supplemented with 1·25 mM $CaCl_2$. After 2 min of perfusion, the perfusion solution was switched to a nominally calcium-free Joklik medium supplemented with 20 mM creatine and 60 mM taurine for 5 min. The same medium supplemented with 1·0 mg ml^{-1} of collagenase (Worthington), 0·1% bovine serum albumin (BSA) (Sigma), and 50 μm $CaCl_2$ was used to perfuse the heart for 4–5 min. The ventricles were then minced and incubated at 37 °C for 15 min in Joklik medium containing 2% BSA with gentle agitation to separate the cells. After two washes, the myocytes were suspended in the same medium containing 1·25 mM $CaCl_2$. All media used during the above procedures were saturated with 5% CO_2–95% O_2. Before the experiments the cells were kept in Tyrode solution at room temperature (22 °C) for 2–6 h. The cells were loaded with fluo-3 by a 20 min incubation with 5 μM fluo-3 AM (acetoxymethyl ester form, Molecular Probes) at 22 °C.

The standard Tyrode solution contained (mM): 140 NaCl, 2 KCl, 0·5 $MgCl_2$, 1 or 10 $CaCl_2$, 10 Hepes, 0·25 NaH_2PO_4, 5·6 glucose, pH 7·3. Tetrodotoxin (10 μM; Sigma) was added to the bathing solution to avoid depolarization-induced Ca^{2+} release due to spontaneous action potentials. All experiments were started in a bathing solution containing 1 mM Ca^{2+}. Only cells that showed no spontaneous waves during a 1 min observation period were selected for further measurements. To induce Ca^{2+} overload, extracellular $[Ca^{2+}]$ ($[Ca^{2+}]_o$) was increased from 1 to 10 mM. Tetracaine (Sigma) was added from a 100 mM stock solution in methanol, at the concentrations needed.

Confocal microscope

Experiments were performed using an Olympus Laser Scanning Confocal Microscope (LSM-GB200) equipped with an Olympus ×60 (1·4 numerical aperture) objective lens. For imaging intracellular $[Ca^{2+}]_i$, the system was operated in the line-scan mode. 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. Images were acquired at a rate of 2·1 or 8·3 ms per scan with the confocal detector aperture set to 10–25% of maximum. Image processing and analysis were performed using NIH Image (NIH, Bethesda, MD, USA) and IDL software (Research Systems Inc., Boulder, CO, USA). For calibration purposes the total line-scan $[Ca^{2+}]_i$ in 1 mM $[Ca^{2+}]_o$ (normal Ca^{2+} load) was assumed to be 100 nM; it served as a reference point for all subsequent measurements performed in the same cells. $[Ca^{2+}]$ changes were calculated from fluo-3 fluorescence using an equation and calibration parameters described previously (Cheng *et al.* 1993). Correction factors obtained *in situ* were used to correct all optical signals recorded in the presence of tetracaine for a small direct inhibition of fluo-3 fluorescence by this agent. The following criteria were applied to identify local Ca^{2+}-release events (Ca^{2+} sparks, Santana, Cheng, Gomez, Cannel & Lederer, 1996): (a) the amplitude of the event had to be at least two times greater than the standard deviation of fluorescence intensity fluctuations measured in the neighbouring region (area $\approx 3 \times 15$ pixels); (b) the duration and image width of the Ca^{2+} signal (both measured at half-maximal amplitude) had to be within 10–100 ms and 0·5–3 μm, respectively.

Preparation of SR membrane vesicles

Heavy SR microsomes were isolated by differential centrifugation from the ventricles of dog heart as described previously (Dettbarn, Györke & Palade, 1994). Dogs were killed by lethal injection of Nembutal. Membrane vesicles were frozen rapidly and stored in liquid nitrogen.

Lipid bilayer experiments

SR microsomes were fused into planar lipid bilayers and single channels were monitored as described previously (Lukyanenko *et al.* 1996). Bilayers were composed of 80% phosphatidylethanolamine and 20% phosphatidylcholine dissolved in decane at a final concentration of 50 mg ml^{-1}. SR vesicles were added to one side of the bilayer (defined as *cis*), and the other side was defined as *trans* (virtual ground). The orientation of the incorporated RyR channels was such that the cytoplasmic side was in the *cis* compartment (Györke, Velez, Suarez-Isla & Fill, 1994). Standard solutions contained 350 mM *cis* $CsCH_3SO_3$, 20 μM *trans* $CsCH_3SO_3$, 20 μM $CaCl_2$, 20 mM Hepes (pH 7·2). After channel incorporation, the *trans* $CsCH_3SO_3$ concentration was adjusted to 350 mM. Single channel recording was performed using a custom current–voltage conversion amplifier (Györke *et al.* 1994). Data were filtered at 1–2 kHz and digitized at 2–5 kHz. Acquisition and analysis of data were performed using pCLAMP 6.01 software (Axon Instruments).

SR Ca^{2+} uptake measurements

Calcium uptake measurements were carried out spectrophotometrically (absorbancies measured at 710 and 790 nm, $A_{710} - A_{790}$) using antipyrylazo III to monitor Ca^{2+} concentration outside the membrane vesicles (Dettbarn *et al.* 1994). The medium in the cuvette consisted of (mM): 100 KCl, 20 K-Mops, 0·25 anti-

pyrylazo III, 1 potassium phosphate, 1 Mg-ATP, 5 disodium phosphocreatine, and 20 $\mu g\ ml^{-1}$ creatine phosphokinase, pCa 4·8, pH 6·95. In addition, to inhibit SR Ca^{2+} release in some experiments the medium was supplemented with 0·1–1 μM Ruthenium Red (Sigma). Membranes (0·5–1·0 mg of protein) were added to the cuvette, and active Ca^{2+} uptake was initiated by administration of 12 nmol $CaCl_2$.

Statistics

Data were expressed as means ± S.E.M. Two-sample comparisons were performed by using Student's unpaired *t* test, and significance was defined at $P < 0·05$.

RESULTS

Effect of tetracaine on spontaneous Ca^{2+} release in Ca^{2+} overloaded myocytes

Figure 1*A* shows line-scan fluo-3 images recorded in three representative cells under control conditions (−1 min) and at various times following addition of three different concentrations of tetracaine (0·5, 0·75 and 1·5 mM). To induce Ca^{2+} overload, Ca^{2+} in the extracellular bathing solution was increased from 1 to 10 mM. In accordance with previous studies (Cheng *et al.* 1993, 1996; Lukyanenko *et al.* 1996), Ca^{2+} overloaded cells under control conditions exhibited multiple spontaneous release events (sparks) and propagating Ca^{2+} waves. When added to the bathing solution, tetracaine, at concentrations above 0·25 mM, inhibited Ca^{2+} waves and Ca^{2+} sparks (Fig. 1*A b*, *B b* and *C b*). Beginning 2–3 min after application of a submaximal tetracaine dose (<1·25 mM), a gradual increase in the frequency of sparks was observed (Fig. 1*A c* and *B c*). At moderate tetracaine concentrations (<0·75 mM) this increase in release activity typically resulted in reappearance of propagating Ca^{2+} waves. Depending on the tetracaine concentrations used, these Ca^{2+} signals varied from large amplitude and high velocity waves (0·25 and 0·5 mM tetracaine, Fig. 1*A d*) to

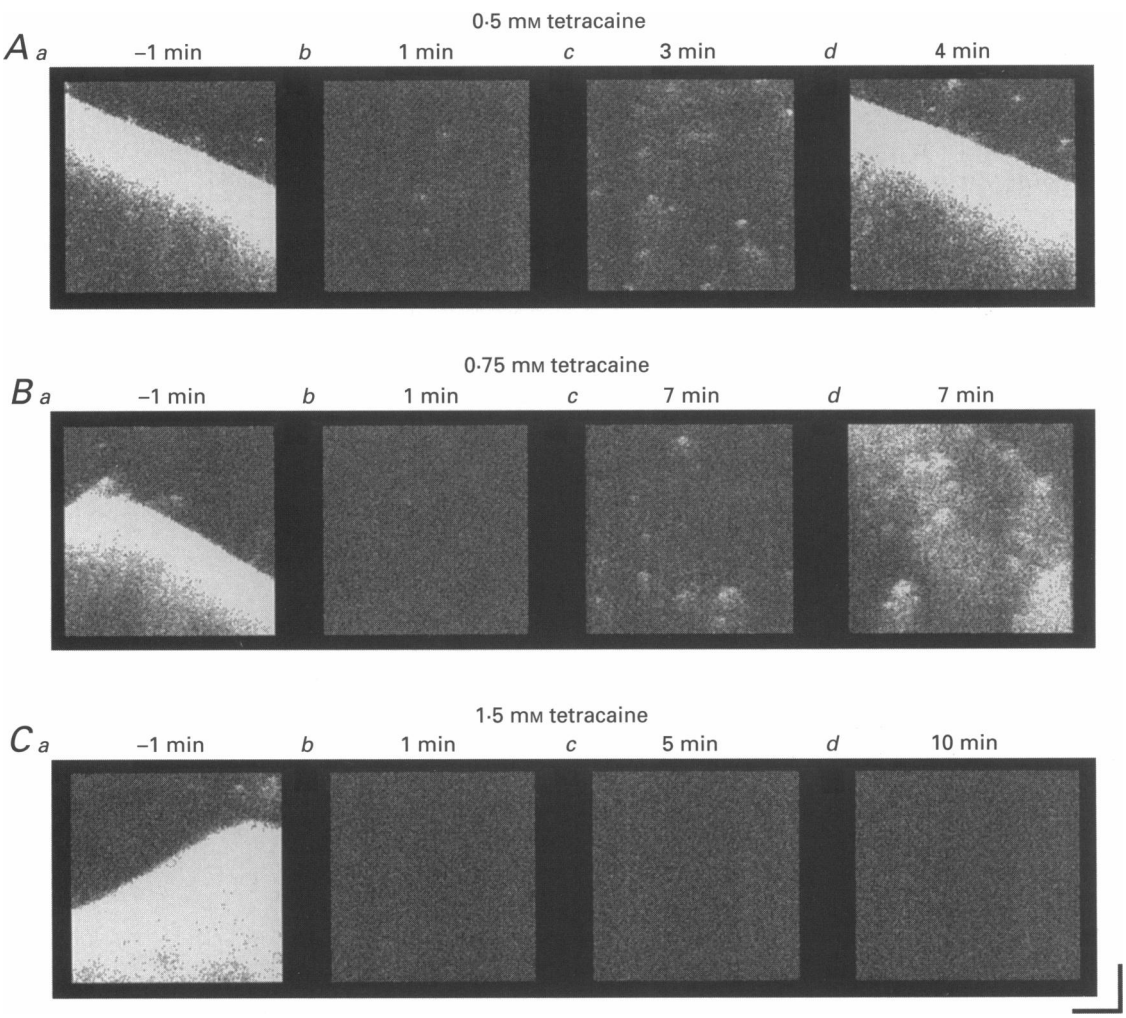

Figure 1. Effect of tetracaine on spontaneous Ca^{2+} release in Ca^{2+}-overloaded rat ventricular myocytes

Line-scan images of $[Ca^{2+}]$ changes acquired under control conditions (10 mM $[Ca^{2+}]_o$) and at different times after addition to the bathing solution of 0·5 (*A*), 0·75 (*B*) and 1·5 mM tetracaine (*C*). The time after application of the drug, which occurred at 0 min, is indicated above the images. Calibration bars: horizontal, 15 μm; vertical, 200 ms.

very slow waves with poorly defined structure (0·5 and 0·75 mM tetracaine, not shown). At still higher concentrations (≥0·75 mM), no propagating waves usually arose; however, spontaneous Ca^{2+} release still could be observed in the form of a non-propagating multifocal process that occurred simultaneously over large areas of the cell (Fig. 1*Bd*). No delayed potentiation of spontaneous release was detected with tetracaine ≥1·5 mM, concentrations that completely inhibited all forms of release during periods of observation of 10–15 min (Fig. 1*Cb–Cd*). These results show that under Ca^{2+} overload conditions submaximal doses of tetracaine exhibit biphasic effects on spontaneous Ca^{2+} release. In the first phase tetracaine inhibits release; in the second phase it potentiates release.

To examine more closely the effects of tetracaine on Ca^{2+} release we quantified the spatiotemporal properties of sparks under control conditions and in the presence of different doses of the drug. The time dependence of the effects of 0·75 mM tetracaine on frequency, amplitude and duration of sparks in a typical experiment is illustrated in Fig. 2*A–C*. One to two minutes after addition of the drug the frequency of sparks was reduced by about 90%, while the magnitude and duration were diminished by approximately 60 and 10%, respectively. Further exposure to the drug resulted in a gradual potentiation of sparks. When measured 5–6 min after addition of the drug, spark frequency, amplitude and duration were increased by about 100, 30 and 90%, respectively, above the control levels. Quantification of sparks at later times was difficult because they began to fuse into widely spread Ca^{2+} elevations where individual events could not be clearly distinguished (Fig. 1*Bd*). The tetracaine dependence of spark frequency, amplitude and duration

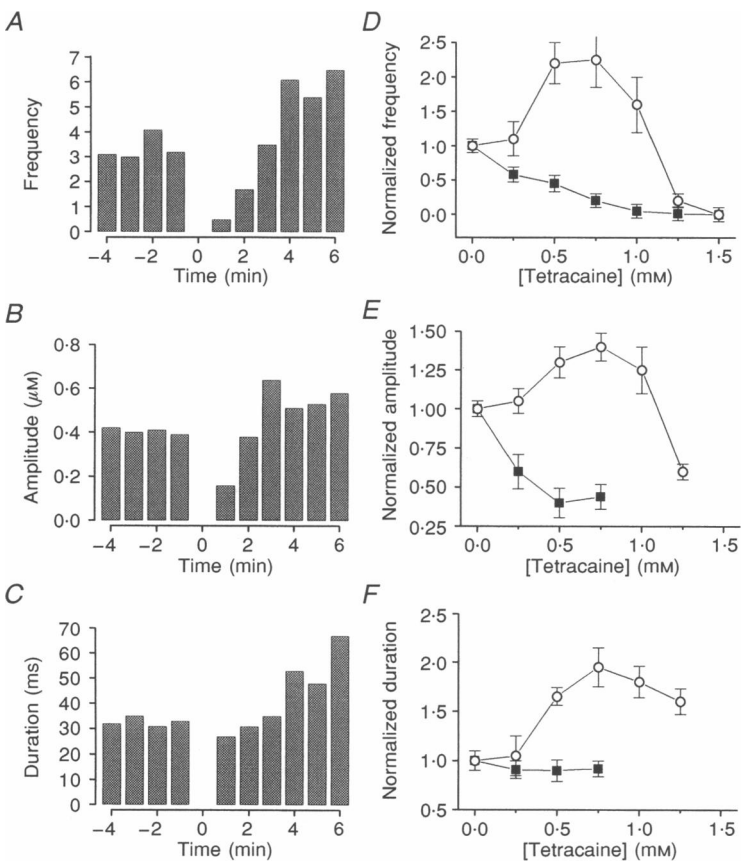

Figure 2. Effect of tetracaine on spatiotemporal properties of Ca^{2+} sparks in Ca^{2+}-overloaded myocytes

A–C, time dependence of the effect of 0·75 mM of tetracaine on frequency, peak amplitude and duration of sparks, respectively. Tetracaine at 0·75 mM was added at time 0 on the horizontal axis. Spark frequency was measured as the number of sparks per second per 100 μm line scanned. Spark amplitude was defined as the difference between the peak $[Ca^{2+}]$ during the spark and the mean $[Ca^{2+}]$ during a 100 ms period prior to onset of the spark. Spark duration was measured at half-maximal amplitude. The values are absolute (*A*) or means (*B* and *C*) obtained from 1–3 consecutive line-scan images in a single cell. *D–F*, dose–response relationships for the effects of tetracaine on frequency, amplitude and duration of sparks, respectively, as measured 1–2 min (■) or 5–6 min (○) after addition of the drug. The values are means ± S.E.M. obtained from 4–8 individual experiments.

measured separately during the initial inhibitory (1–2 min) and delayed potentiatory phases (5–6 min) is shown in Fig. 2*D–F*. During the initial inhibitory phase, a gradual depression of sparks by increasing tetracaine concentrations is indexed by a decrease in the frequency and amplitude of the events, although the change in spark duration was not significant. Delayed potentiation of sparks occurred at tetracaine concentrations between 0·5 and 1 mM. Higher concentrations resulted in a drastic reduction of spark frequency and magnitude. Sparks were completely abolished by 1·5 mM tetracaine. Taken together, these experiments show that tetracaine has a dual effect on the Ca^{2+} release mechanism. The inhibition of Ca^{2+} sparks is consistent with the blocking effect of tetracaine on Ca^{2+}-release channels (O'Brien *et al.* 1995). The delayed potentiation of release events by submaximal tetracaine concentrations could be mediated by the increase in SR Ca^{2+} content known to be caused by local anaesthetics (Stephenson & Wendt, 1986).

Changes in SR Ca^{2+} content in the presence of tetracaine

To test the possibility that tetracaine enhances SR Ca^{2+} accumulation, caffeine was applied under control conditions and in the presence of the drug (Lukyanenko *et al.* 1996). Figure 3*A* shows representative line plots of time-dependent changes of $[Ca^{2+}]$ induced by 10 mM caffeine measured in two different cells before and after (2 and 5 min) addition of 0·75 mM tetracaine. It can be seen that after 2 min of exposure to tetracaine, the magnitude of the Ca^{2+} transient increased about 20%. Longer (5 min) exposure resulted in an even larger increase in the caffeine-induced Ca^{2+} transients (~50%). The results of this series of experiments are summarized in Fig. 3*B*, which compares the amplitudes of caffeine-induced Ca^{2+} transients measured under control conditions and following 2 or 5 min of exposure to 0·75 mM tetracaine. As indexed by these changes in the caffeine-induced Ca^{2+} transients, continuous exposure of the cells to the drug for 2 or 5 min resulted in 19 and 54% increase in the SR Ca^{2+} load, respectively. These results suggest that tetracaine causes a progressive increase in Ca^{2+} accumulation inside the SR.

Dependence of tetracaine effects upon extracellular Ca^{2+} concentration

Sarcoplasmic reticulum Ca^{2+} load of cardiomyocytes is known to relate to the levels of Ca^{2+} in the extracellular medium (Stern *et al.* 1988). To further evaluate the possibility that delayed potentiation of spontaneous Ca^{2+} release by tetracaine is due to an increased SR Ca^{2+} load, we explored the reliance of this phenomenon on extracellular

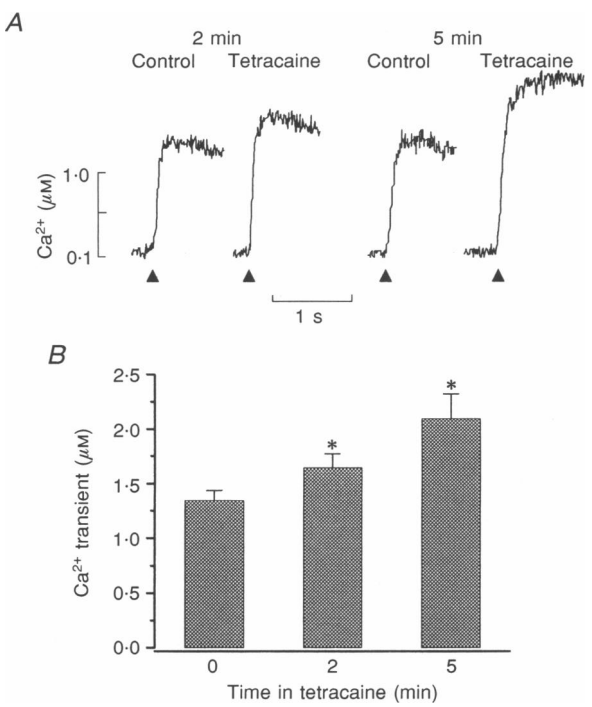

Figure 3. The effect of tetracaine on SR Ca^{2+} load

A, caffeine-induced Ca^{2+} transients measured in two different cells before, and 2 or 5 min after, addition of 0·75 mM tetracaine to the bathing solution, which contained 10 mM Ca^{2+}. Addition of caffeine (10 mM) is indicated by arrowheads. *B*, amplitude of caffeine-induced Ca^{2+} transients for different times of continuous exposure of the cells to 0·75 mM tetracaine. The values are means ± S.E.M obtained from 7–19 individual experiments. $*P < 0·05$ *vs.* 0 min in tetracaine.

Ca^{2+} concentration. Figure 4 shows representative line-scan fluo-3 images of cells exposed to 1 (*A*) or 0·5 mM Ca^{2+} (*B*) measured before and after addition of 0·75 mM tetracaine. Histograms of spark frequency are also shown below the images. Before addition of the drug, the cells in both 1 and 0·5 mM Ca^{2+} exhibited occasional sparks but no spontaneous waves. Similar to the experiments performed at 10 mM $[Ca^{2+}]_o$, addition of 0·75 mM tetracaine inhibited all sparks. At 1 mM $[Ca^{2+}]_o$, following this initial inhibition, the sparking activity reappeared and increased over time, but at a much slower rate than in experiments with 10 mM $[Ca^{2+}]_o$ (Fig. 2*A*). The time needed to attain a frequency that was 50% of the control level was 5·5 ± 0·6 min ($n = 6$) compared with 2 ± 0·5 min ($n = 9$, $P < 0·05$) in 10 mM Ca^{2+}. In 0·5 mM Ca^{2+} no measurable increase in sparking activity was observed during an observation period of 10 min in the presence of tetracaine (Fig. 4*B*). Similar results were obtained in four other experiments. These results suggest that increased SR Ca^{2+} accumulation is essential for the development of the delayed potentiation of spontaneous Ca^{2+} release.

Effect of tetracaine on SR Ca^{2+} uptake and sarcolemmal Ca^{2+}-extrusion mechanisms

The observed changes in $[Ca^{2+}]_i$ in the presence of tetracaine could be attributed to an inhibition by the drug of the cellular Ca^{2+}-removal mechanisms. To assess the effects of tetracaine on Ca^{2+} removal by sarcolemmal Ca^{2+}-transport

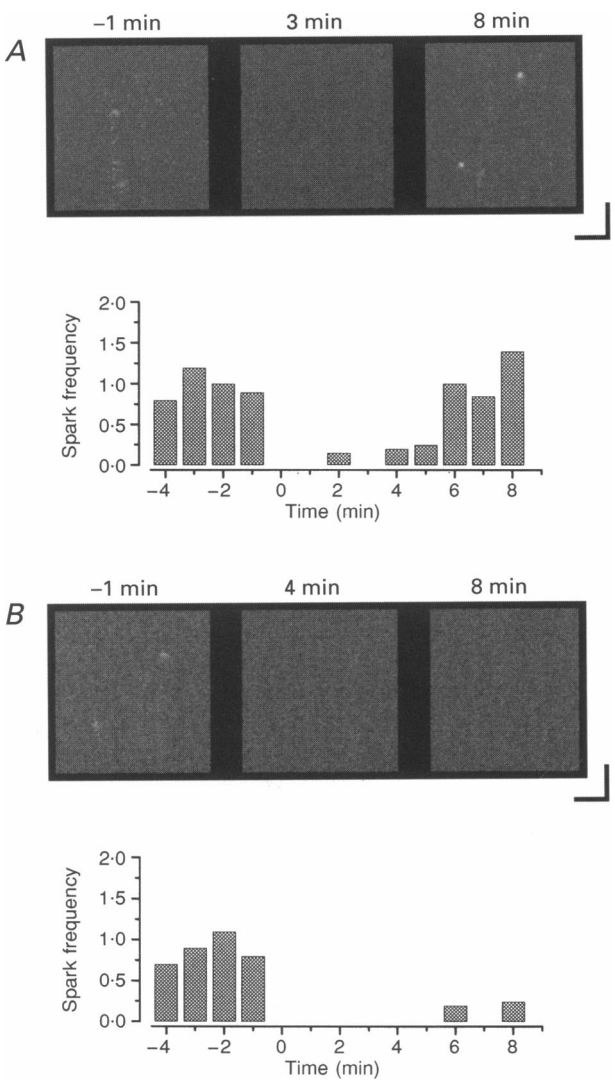

Figure 4. Time dependence of the effects of tetracaine on Ca^{2+} sparks in myocytes exposed to low extracellular Ca^{2+}

The extracellular solution contained 1 mM (*A*) or 0·5 mM (*B*) Ca^{2+}. Line-scan images (top) of Ca^{2+} changes acquired before (−1 min) and after administration of 0·75 mM tetracaine at the times indicated above the images. Calibration bars: horizontal, 10 μm; vertical, 200 ms. Frequency of sparks as a function of time (bottom) was measured before and after addition of the drug. Spark frequency was determined as the number of sparks per second per 100 μm line scanned, from 1–3 consecutive line-scan images. Tetracaine (0·75 mM) was added at time 0.

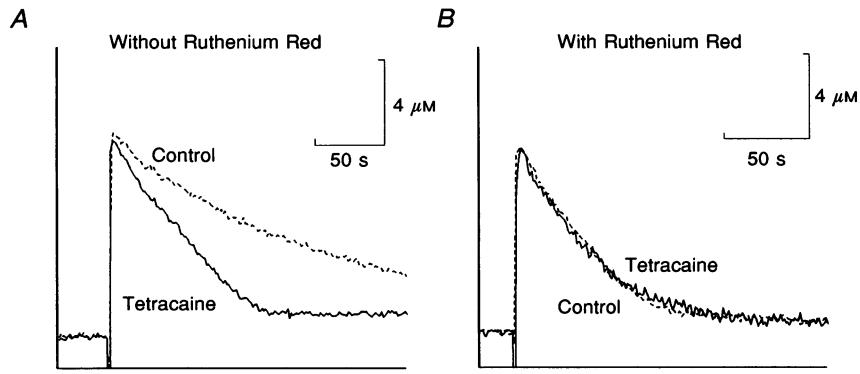

Figure 5. Effect of tetracaine on Ca^{2+} uptake by cardiac microsomal membrane preparations
Ca^{2+} uptake measured in the absence (*A*) and presence (*B*) of 1 μM Ruthenium Red. Canine cardiac microsomes (600 μg of protein) were administered 12·5 nmol of $CaCl_2$ under control conditions (dashed traces) and in the presence of 1 mM tetracaine (continuous traces), all in the presence of 1 mM phosphate and 1 mM Mg-ATP. Measurements in *B* were performed in the presence of 1 μM Ruthenium Red. The traces are representative of 9–12 separate measurements in 3 different membrane preparations.

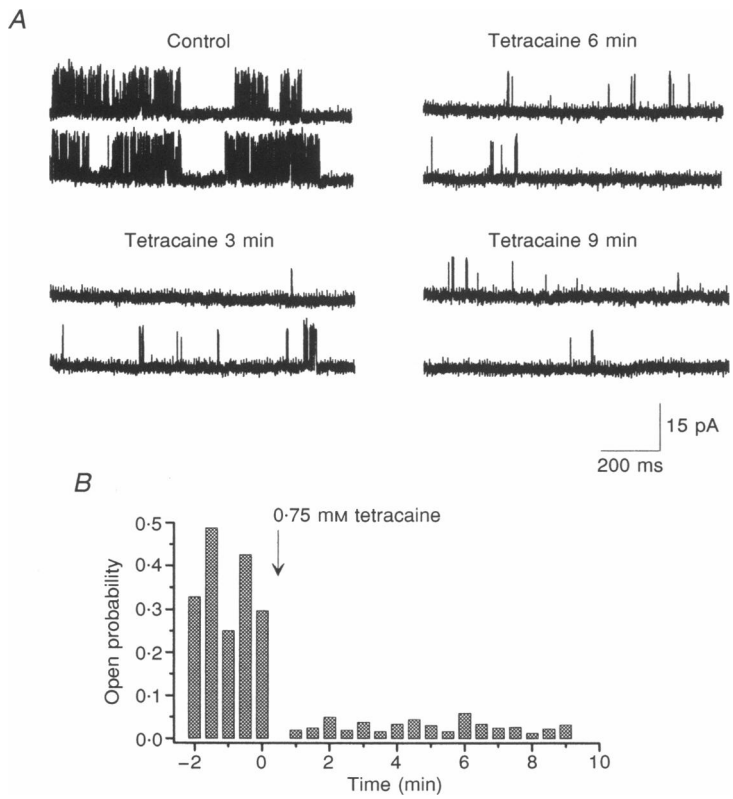

Figure 6. Effect of tetracaine on the activity of single cardiac Ca^{2+}-release channel during a continuous recording of 10 min
A, single-channel currents recorded under the control conditions and at different times (3, 6 and 9 min) following addition of 0·75 mM tetracaine to the *cis* chamber. *B*, channel open probability as a function of the time before, and after, addition of the drug. Single-channel openings are shown as upward deflections. *Cis* chamber contained 350 mM $CsCH_3SO_3$, 3 mM ATP, 3 μM free Ca^{2+}, pH 7·2; *trans* chamber contained 350 $CsCH_3SO_3$, pCa 4·7, pH 7·2. Tetracaine (0·75 mM) was added to the *cis* chamber. Holding potential was 30 mV.

mechanisms (i.e. Ca^{2+} pump and Na^+–Ca^{2+} exchanger) we recorded intracellular fluo-3 fluorescence in cells in which the Ca^{2+} gradient across the SR membrane had been abolished by 10 μM ryanodine. No significant alteration in $[Ca^{2+}]_i$ was detected in 10 min of continuous exposure of the cells to 1 mM tetracaine. The $[Ca^{2+}]_i$ measured before and 5 or 10 min after administration of the drug was 108 ± 5, 111 ± 7 and 109 ± 9 nM (means ± S.E.M., $n = 5$), respectively.

To assess the effects of tetracaine on Ca^{2+} removal by the SR Ca^{2+} pump, SR Ca^{2+}-uptake measurements were performed spectrophotometrically in isolated cardiac microsomal preparations using antipyrylazo III. The net Ca^{2+} uptake was not inhibited but was significantly enhanced in the presence of 1 mM tetracaine (Fig. 5*A*). This potentiatory effect of the drug on Ca^{2+} accumulation was completely eliminated when the RyR channels had been blocked by 1 μM Ruthenium Red prior to the addition of 1 mM tetracaine. As shown in Fig. 5*B*, under these conditions tetracaine had virtually no effect on Ca^{2+} uptake. These results suggest that: (1) tetracaine (1 mM) does not have a direct inhibitory effect on Ca-ATPase-mediated Ca^{2+} uptake, and (2) it can potentiate net SR Ca^{2+} accumulation by preventing leakage of Ca^{2+} through the RyR channels. Based on these results, we conclude that the observed potentiation of Ca^{2+} release by tetracaine in intact myocytes is not due to an inhibition of the Ca^{2+}-transport mechanisms that remove Ca^{2+} from the cytoplasm.

Effect of tetracaine on single Ca^{2+}-release channels

To visualize the effects of tetracaine on the Ca^{2+}-release mechanism more directly, we performed measurements of single cardiac SR Ca^{2+}-release channels (RyRs) inserted into lipid bilayers. Channels were activated by 3 μM Ca^{2+} (free) and 3 mM ATP (total) and channel activity was monitored

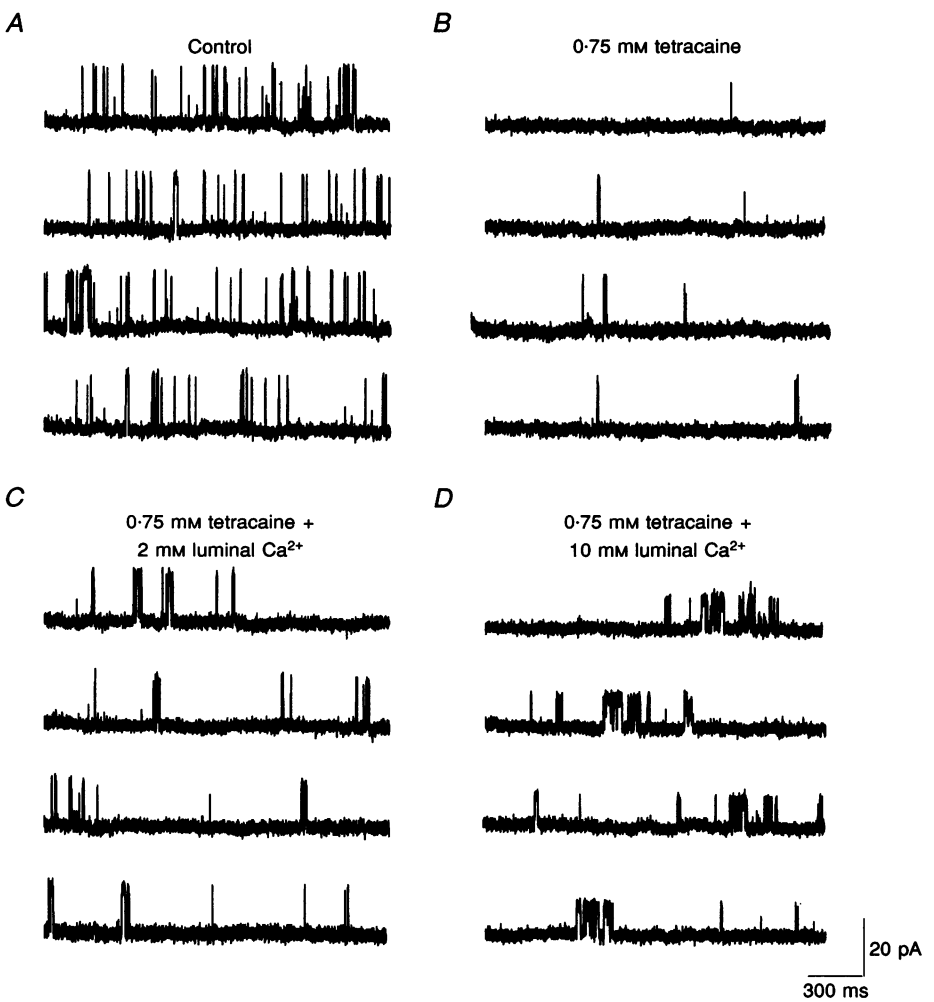

Figure 7. Effect of luminal Ca^{2+} on a cardiac SR Ca^{2+}-release channel inhibited by tetracaine

Current fluctuations measured through a single cardiac Ca^{2+}-release channel (RyR) under control conditions (*cis*: 3 mM ATP, pCa 5·5; *trans*: pCa 4·7; *A*), 3 min following addition of 0·75 mM tetracaine to the *cis* chamber (*B*), and 3 min after addition of 2 mM (*C*) or 10 mM (*D*) Ca^{2+} to the *trans* chamber. Single-channel openings are shown as upward deflections. Holding potential was 30 mV.

using Cs^+ as the charge carrier. Figure 6*A* shows representative recordings of a cardiac RyR under control conditions and at various times (3, 6 and 9 min) after addition of 0·75 mM tetracaine to the *cis* chamber. Channel open probability (P_o) during the course of the experiment is plotted in Fig. 6*B*. It can be seen that, upon its addition, tetracaine reduced channel P_o by about tenfold and that the P_o of the inhibited channel remained relatively stable during a 10 min period of continuous recording. Similar results were obtained in six other channels. These results suggest that the delayed potentiation of Ca^{2+} release observed in intact myocytes (Fig. 1*A* and *B*) is not a direct result of interaction of tetracaine with the Ca^{2+}-release channel.

Increasing luminal Ca^{2+} has been shown to enhance the activity of the SR Ca^{2+}-release channels activated by cytoplasmic Ca^{2+} and ATP (Lukyanenko *et al.* 1996). To investigate whether luminal Ca^{2+} has a similar impact on channels affected by tetracaine, we performed single-channel measurements in the presence of various tetracaine concentrations before and after elevation of *trans* Ca^{2+} from 20 μM to 2 mM and 10 mM. As seen in Fig. 7*B*, with a channel attenuated by 0·75 mM tetracaine, elevation of Ca^{2+} in the *trans* chamber resulted in a marked increase in channel activity (Fig. 7*C* and *D*). The primary effect of luminal Ca^{2+} was to increase the number of openings (Table 1). A 36% increase (not significant) in the mean duration of the open events was also detected. In addition, in the presence of 10 mM luminal Ca^{2+}, unitary Ca^{2+} currents were reduced as Ca^{2+} competed with Cs^+, the primary charge-carrying ion (Tu, Velez, Cortez-Gutierrez & Fill, 1994). Dose–response relationships for the reduction by tetracaine of channel P_o at low and high luminal [Ca^{2+}] are shown in Fig. 8. Pooled data from a total of twenty-one experiments are presented. Besides increasing P_o at all submaximal blocking concentrations of the drug, increased luminal Ca^{2+} resulted in a significant reduction of channel sensitivity to tetracaine. Data for 20 μM and 10 mM luminal Ca^{2+} were best fitted by the theoretical curves with EC_{50} values of 0·26 ± 0·03 mM ($n = 5$) and 0·65 ± 0·12 mM ($n = 7$, $P < 0·05$), respectively. These results suggest that high luminal Ca^{2+} potentiates the activity of the SR Ca^{2+} release channels by: (1) enhancing channel activity in a manner similar to that in the absence of the drug, and (2) removal of the inhibitory action of tetracaine.

DISCUSSION

The principal finding of this study is that submaximal blocking concentrations of tetracaine exert biphasic effects on spontaneous SR Ca^{2+} release in cardiac myocytes. In the initial phase of its action, tetracaine inhibited spontaneous release in all its forms. In the second phase of its action, tetracaine led to potentiation of spontaneous Ca^{2+} release, as manifested by an increase in the frequency and magnitude of sparks and generation of a spectra of large scale signals, ranging from propagating Ca^{2+} waves to non-propagating multifocal Ca^{2+} releases. The initial inhibitory action of tetracaine on Ca^{2+} release is consistent with the blocking effect of the drug on Ca^{2+}-release channels (Meissner & Henderson, 1987; O'Brien *et al.* 1995; and the present study). Elucidation of the delayed potentiatory effect of tetracaine was the primary concern of the present study. Our basic conclusion is that the delayed potentiation of spontaneous Ca^{2+} release by tetracaine is due to a further

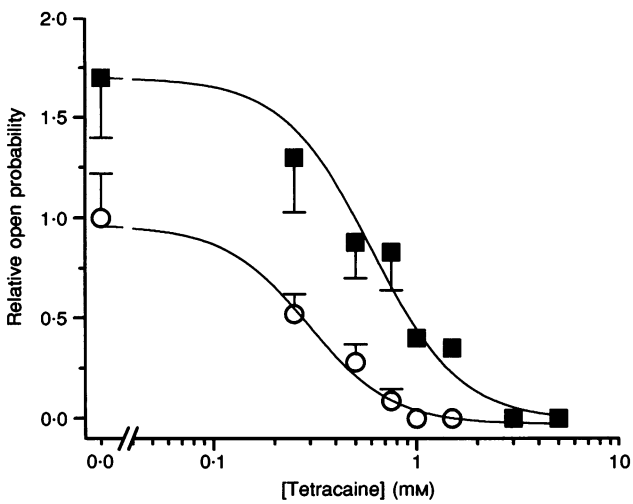

Figure 8. The dose–response relationship for tetracaine reduction of cardiac SR Ca^{2+}-release channel open probability measured at low (pCa 4·7, ○) and high luminal Ca^{2+} (pCa 2, ■)

The open probability is normalized to that in low luminal Ca^{2+} and in the absence of tetracaine. Where error bars are given they represent S.E.M. of three or more experiments. The continuous curves were obtained from the equation: $P_{rel} = 1/(1 + ([\text{tetracaine}]/EC_{50})^p)$, with $EC_{50} = 0·26$ mM and $p = 1·89$ for *trans* pCa 4·7, and $EC_{50} = 0·65$ mM and $p = 2·2$ for *trans* pCa 2.

increase in SR Ca^{2+} load by the drug and the concomitant activation of the Ca^{2+}-release channels by the elevated luminal Ca^{2+}.

This conclusion is supported by the following evidence: (1) exposure to tetracaine caused an increase in Ca^{2+} accumulation within the SR in intact myocytes (Fig. 3) as well as in isolated membrane preparations (Fig. 5); (2) the potentiatory effect of tetracaine depended critically on $[Ca^{2+}]$ in the extracellular medium and thus, on the capability of the cells to accumulate Ca^{2+} inside the SR (Fig. 4); (3) increasing Ca^{2+} at the luminal side of single Ca^{2+}-release channels (RyRs) in bilayers resulted in an increase in channel P_o under control conditions as well as in the presence of various concentrations of tetracaine (Fig. 8).

The following alternative possibilities for delayed potentiation of spontaneous Ca^{2+} release were considered and ruled out based on the results of our experiments: (1) elevation of cytosolic $[Ca^{2+}]$ via inhibition by tetracaine of cellular Ca^{2+}-removal mechanisms (i.e. SR and sarcolemmal Ca-ATPases, Na^+–Ca^{2+} exchange); (2) induction of Ca^{2+} release through pathways other than the SR Ca^{2+}-release channels; (3) direct activation by tetracaine of SR Ca^{2+}-release channels. The role of inhibition of the SR Ca^{2+} uptake was ruled out in direct measurements of active Ca^{2+} uptake in isolated SR preparations (Fig. 5). Similarly, no significant change in cytoplasmic $[Ca^{2+}]$ was observed in cells treated with ryanodine following exposure to tetracaine, indicating that sarcolemmal Ca^{2+}-removal mechanisms (i.e. Ca^{2+} pump and Na^+–Ca^{2+} exchanger) were not considerably affected by tetracaine under the conditions of our experiments. The possibility that release was induced through pathways other than the SR Ca^{2+}-release channels is not likely, as the effect of tetracaine was clearly manifested by an increase in the frequency and magnitude of Ca^{2+} sparks, events that are believed to be associated with the openings of SR Ca^{2+}-release channels. Furthermore, the potentiating effects of tetracaine were limited to concentrations below 1·5 mM; at higher concentrations tetracaine fully inhibited all forms of release (Figs 1 and 2). If there was a tetracaine-induced Ca^{2+}-release mechanism, increases in tetracaine concentration would be expected only to enhance, but not inhibit, Ca^{2+} release. The same argument applies to the possibility that release potentiation was due to a direct activation of the Ca^{2+}-release channels by tetracaine. In addition, the possibility that release channels were activated by tetracaine in a direct manner is inconsistent with the results of our single-channel experiments, which showed no time-dependent increase in the activity of channels exposed to tetracaine (Fig. 6). Taken together, these results suggest that delayed potentiation of release by tetracaine is due to an increase in SR Ca^{2+} load in the presence of the drug and subsequent activation of the release channels by elevated Ca^{2+} inside the SR.

The demonstrated increase in SR Ca^{2+} load in the presence of tetracaine in intact myocytes is consistent with the study by Stephenson & Wendt (1986) showing an increase in SR Ca^{2+} accumulation in skinned cardiac cells in buffered Ca^{2+} solutions containing procaine. Inhibition of the Ca^{2+} efflux through Ca^{2+}-release channels by these local anaesthetics may account for, or contribute to, a greater net Ca^{2+} accumulation. In line with this possibility, the potentiatory effect of tetracaine on Ca^{2+} accumulation in cardiac microsomal preparations was removed by inhibition of the RyR channels with Ruthenium Red (Fig. 5). Another mechanism whereby tetracaine could enhance SR Ca^{2+} accumulation involves the ability of local anaesthetics to block the SR K^+ channels. It has been shown that a variety of SR K^+-channel blockers including procaine are able to increase the amount of releasable Ca^{2+} significantly in skinned amphibian muscle fibres (Fink & Stephenson, 1987; Fink & Veigel, 1996). The mechanism of action of the K^+-channel blockers on SR Ca^{2+} load presumably involves indirect modulation of Ca^{2+} binding sites within the SR lumen through counter-currents for H^+ and Mg^{2+} ions (Fink & Stephenson, 1987; Fink & Veigel, 1996). In principle, the increase in SR Ca^{2+} accumulation could be also due to the reported ability of tetracaine to inhibit the sarcolemmal Ca^{2+} pump and the Na^+–Ca^{2+} exchange (Gill, Grollman & Kohn 1981; Takuma, Kuyatt & Baum, 1985). However, we detected no increase in cytoplasmic $[Ca^{2+}]$ in ryanodine-treated myocytes, indicating that the sarcolemmal Ca^{2+}-extrusion mechanisms were not significantly inhibited under conditions of our experiments. Furthermore, inhibition of sarcolemmal Ca^{2+}-transport mechanisms clearly could not be responsible for the increase in SR Ca^{2+} accumulation in isolated SR membrane vesicles (present study) and skinned cardiac cells (Stephenson & Wendt, 1986), also implying that inhibition of sarcolemmal Ca^{2+}-transport mechanisms is not the principle explanation for the enhancement of Ca^{2+} accumulation by these drugs.

The results of our single channel experiments in bilayers confirm those of previous studies, showing that luminal Ca^{2+} increases the activity of cardiac SR Ca^{2+}-release channels (Sitsapesan & Williams, 1994; Lukyanenko *et al.* 1996). An important new finding reported here is that the relative potentiatory effect of luminal Ca^{2+} on channel P_o was even further enhanced in the presence of tetracaine. At elevated luminal Ca^{2+}, the dose–response relation for P_o inhibition by tetracaine was shifted to higher drug concentrations (Fig. 8). Thus, it appears that the luminal Ca^{2+}-induced augmentation of channel activity in the presence of tetracaine is due, not only to the effects of luminal Ca^{2+} seen in the absence of the drug but also, to a certain extent, to a removal of the inhibitory action of the drug. The mechanisms of action of luminal Ca^{2+} on channel activity have not been clearly established. One possibility, elaborated for the skeletal RyR is that luminal Ca^{2+} has access to the cytoplasmic activation site of the channel (Tripathy & Meissner, 1996; Herrmann-Frank & Lehmann-Horn, 1996). Another possibility is that the effect of luminal Ca^{2+} is mediated by Ca^{2+} acting at specific sites on the luminal side of the channel (Sitsapesan & Williams, 1994). Finally, in a combination of the first and second

Table 1. The effects of tetracaine and luminal Ca^{2+} on RyR channel gating

Tetracaine (mM)	0	0·75	0·75	0·75
Luminal Ca^{2+} (mM)	0·02	0·02	2	10
Number of events	3597 ± 687	319 ± 114	1516 ± 275*	2796 ± 454*
Open probability (P_o)	0·096 ± 0·015	0·008 ± 0·002	0·044 ± 0·008*	0·093 ± 0·019*
Mean open time (ms)	4·3 ± 0·9	3·9 ± 0·6	4·7 ± 1·0	5·3 ± 1·2
Mean closed time (ms)	41·4 ± 7·9	497·9 ± 77·8	94·2 ± 43·7*	51·9 ± 18·1*

Channel parameters were obtained from 1·6 min continuous recordings as described in Methods. Data recorded as means ± S.E.M. of 4–8 determinations from different experiments. *$P < 0·05$ *vs.* values at 0·75 mM tetracaine and 0·02 mM luminal Ca^{2+}.

mechanisms, the channel could become sensitized to cytosolic Ca^{2+} as a result of allosteric interactions between intraluminal and cytosolic Ca^{2+} sensing sites (Lukyanenko *et al.* 1996). Since it is known that the sensitivity of the channel to local anaesthetics (i.e. procaine) is not affected by *cis* (cytosolic) Ca^{2+} (Zahradnikova & Palade, 1993), the observed modulation of tetracaine sensitivity of the channel by luminal Ca^{2+} should be mediated by sites distinct from the cytoplasmic activation site. Furthermore, the possibility that luminal Ca^{2+} has access to the cytoplasmic activation site is not supported by the observation that luminal Ca^{2+} activated the channel primarily by increasing the frequency of events (Table 1). Indeed, luminal Ca^{2+} could reach the cytoplasmic activation site only when the channel opens and Ca^{2+} can flow through the pore. Once the channel closes, the Ca^{2+} gradient near the mouth of the channel dissipates very rapidly (microseconds; Stern, 1992), making rebinding of Ca^{2+} to the cytoplasmic activation site unlikely. Thus, flow of luminal Ca^{2+} would be expected to have little impact on the frequency of resolvable events. Therefore, our results are consistent with the existence of specific binding sites on the luminal face of the channel that are involved in the effects of luminal Ca^{2+} (the second and third mechanisms above).

Ryanodine receptor-gating changes in the presence of tetracaine and high luminal Ca^{2+} correspond with logical alterations in the properties of Ca^{2+} sparks caused by the drug. The dependence of spark frequency on tetracaine concentration in intact cells was consistent with the tetracaine dependence of single channel P_o in bilayer experiments ($EC_{50} \approx 0·5$–1 *vs.* $EC_{50} \approx 0·3$–$0·6$ mM, Figs 2*D* and 8). Thus, the inhibition of the SR Ca^{2+}-release channel by tetracaine *in situ* appears to be similar to that *in vivo*. The delayed potentiation of release events associated with increased SR Ca^{2+} load in tetracaine-treated myocytes correlated with the reversal of tetracaine inhibition of single-release channels by increased luminal Ca^{2+} (Figs 7 and 8). Unfortunately, the precise free intra-SR $[Ca^{2+}]$ either in normal, or in Ca^{2+}-overloaded cardiac myocytes is not known. The upper limit for $[Ca^{2+}]_{SR}$ is imposed by thermodynamic limitations of the SR Ca^{2+} pump. Based on the estimated values for the free energy change of ATP hydrolysis in cardiac muscle ($\Delta G_{ATP} \approx 62$ kJ mol^{-1}; Allen, Morris, Orchard & Pirolo, 1985), it is probably close to 2–3 mM. Indeed, to establish and maintain a gradient of $[Ca^{2+}]_i$–$[Ca^{2+}]_{SR}$ of 100 nM–3 mM, the SR Ca^{2+} pump would be required to utilize about 81% of ΔG_{ATP} ($\Delta G = 2RT \ln\{[Ca^{2+}]_{SR}/[Ca^{2+}]_i\} \approx 50$ kJ mol^{-1}), where R is the universal gas constant and T absolute temperature, and which is at the limit of efficiency of a Ca^{2+} pump. Thus, luminal (*trans*) Ca^{2+} concentrations of 2 and 10 mM used in our experiments should be considered as an upper limit to the estimations of $[Ca^{2+}]_{SR}$. Importantly, 2 mM *trans* Ca^{2+} reversed the inhibition of the channel by tetracaine almost as effectively as 10 mM *trans* Ca^{2+} (Table 1), suggesting that at 2 mM the effect of luminal Ca^{2+} is close to saturation. Further experiments, however, are needed to define better the correlation between the effects of luminal Ca^{2+} on Ca^{2+} release *in vivo* and *in vitro*.

Although it is generally believed that sparks are the consequence of Ca^{2+} release events, it is not clear whether these signals arise from the openings of one RyR channel or the concerted openings of many channels. In this study, the demonstrated ability of tetracaine to reduce spark magnitudes below control levels clearly shows that sparks are not due to activation of a single channel or a non-reducible cluster of channels, implying a multi-channel origin of sparks. A similar conclusion has been reached recently by Lipp & Niggli (1996), who showed that Ca^{2+} release induced by photolysis of caged Ca^{2+} is spatially homogeneous, suggesting elementary release events (quarks) that are much smaller then sparks. In addition, elementary events 5–10 times smaller than cardiac sparks have been seen in skeletal muscle (Tsugorka, Rios & Blatter, 1995).

As mentioned in the Introduction, initiation of spontaneous Ca^{2+} release could be due to Ca^{2+} acting on a cytoplasmic site of the release channel in a manner similar to that during normal E–C coupling, or to Ca^{2+} acting from inside the SR when the Ca^{2+} content of this organelle becomes sufficiently elevated. In intact myocytes, we showed that, with the Ca^{2+}-release blocker tetracaine, we can reach certain levels of SR Ca^{2+} load that result in 'paradoxical' activation of Ca^{2+} release. In such Ca^{2+} 'superloaded' myocytes, the ability of Ca^{2+} release to overcome the

inhibitory action of tetracaine is in agreement with the results of Fabiato (1992) in skinned cardiac cells. He showed that spontaneous Ca^{2+} release induced by high SR Ca^{2+} load can occur under conditions when the process of CICR is inactivated by elevated bathing [Ca^{2+}]. These results indicate that initiation of spontaneous Ca^{2+} release is mediated by mechanisms substantially different from CICR. A mechanism suggested by the results of our lipid bilayer experiments is that elevation of SR Ca^{2+} load causes the Ca^{2+}-release channels to open via Ca^{2+} acting at high concentrations at specific Ca^{2+} sensing sites on the luminal side of the channel.

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