Measurement of sarcoplasmic reticulum Ca^{2+} content and sarcolemmal Ca^{2+} fluxes in isolated rat ventricular myocytes during spontaneous Ca^{2+} release

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1. Intracellular calcium concentration ($[Ca^{2+}]_i$) and Na^+–Ca^{2+} exchange currents were measured in calcium-overloaded voltage-clamped rat ventricular myocytes loaded with the Ca^{2+}-sensitive fluorescent indicator indo-1. Sarcoplasmic reticulum (SR) Ca^{2+} content was measured from the integral of the caffeine-evoked current. In cells that had spontaneous SR Ca^{2+} release in 1 mM external Ca^{2+} concentration ($[Ca^{2+}]_o$), raising $[Ca^{2+}]_o$ increased the frequency of release with no effect on SR Ca^{2+} content. In quiescent cells, increased $[Ca^{2+}]_o$ produced spontaneous Ca^{2+} release associated with increased SR Ca^{2+} content. Further increase of $[Ca^{2+}]_o$ had no effect on SR Ca^{2+} content. The amount of Ca^{2+} leaving the cell during each release was constant over a wide range of frequencies and $[Ca^{2+}]_o$ values. It appears there is a maximum level of SR Ca^{2+} content, perhaps because spontaneous Ca^{2+} release results when the content reaches a threshold.

2. From the relationship between $[Ca^{2+}]_i$ and Na^+–Ca^{2+} exchange current during a caffeine response, it is possible to estimate the changes in Na^+–Ca^{2+} exchange current expected from a change of $[Ca^{2+}]_i$. The data show that the calcium oscillations contribute a significant fraction of the total extra Ca^{2+} efflux induced by increasing $[Ca^{2+}]_o$. Raising $[Ca^{2+}]_o$ decreased the rate of calcium removal from the cell as measured from the rate of decay of the caffeine response, suggesting that both inhibition of Ca^{2+} efflux and increased Ca^{2+} entry account for the Ca^{2+} overload at elevated $[Ca^{2+}]_o$.

3. Inhibiting spontaneous SR Ca^{2+} release increases resting $[Ca^{2+}]_i$. The Ca^{2+} efflux is identical to that in the presence of release. It is concluded that spontaneous release of calcium, although potentially arrhythmogenic, is an effective way to activate Ca^{2+} efflux in overloaded conditions and minimizes any increase of diastolic tension.

Under conditions that increase the calcium load of cardiac cells (e.g. in the presence of cardiac glycosides or elevated external calcium concentration), resting intracellular calcium concentration ($[Ca^{2+}]_i$) increases and spontaneous waves of increased calcium and contraction appear (Orchard, Eisner & Allen, 1983; Stern, Kort, Bhatnagar & Lakatta, 1983; Wier, Kort, Stern, Lakatta & Marban, 1983; Wier, Cannell, Berlin, Marban & Lederer, 1987). These waves result from cyclical release and re-uptake of calcium by the sarcoplasmic reticulum (SR). Although in cardiac muscle such waves of SR Ca^{2+} release are not seen under normal conditions, in other tissues they are a normal mechanism of cellular signalling (Kasai & Petersen, 1994).

The aim of this paper was to investigate the following questions. (i) In calcium overload, how 'overloaded' is the sarcoplasmic reticulum? In other words, what is the change of SR Ca^{2+} content that accompanies the development of spontaneous SR Ca^{2+} release and, as the cell becomes more 'Ca^{2+} overloaded' and the frequency of spontaneous SR release increases, what happens to SR Ca^{2+} content? (ii) How much calcium efflux is activated by the elevation of $[Ca^{2+}]_i$ during spontaneous release? (iii) In calcium overload, what is the relative quantitative importance of the calcium wave and elevated resting $[Ca^{2+}]_i$ in stimulating Ca^{2+} pumping out of the cell? These points have been studied by making use of the fact that Ca^{2+} removal from the cell during spontaneous oscillations of $[Ca^{2+}]_i$ can be quantified by measuring the resulting Na^+–Ca^{2+} exchange current (Callewaert, Cleemann & Morad, 1989; Varro, Negretti, Hester & Eisner, 1993; Negretti, Varro & Eisner, 1995). This, in turn, can be compared with the SR Ca^{2+} content estimated from the current produced by releasing SR Ca^{2+} with caffeine. The results show that, once a Ca^{2+}-overloaded state is reached,

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further overload has no effect on SR Ca^{2+} content. In addition, a large fraction of the Ca^{2+} removal from the cell occurs during the waves and this is varied primarily by changes in the frequency of the waves rather than their magnitude.

METHODS

Experiments were carried out on cardiac myocytes isolated from rat ventricles using a collagenase and protease digestion protocol as previously described (Eisner, Nichols, O'Neill, Smith & Valdeolmillos, 1989). Rats were killed by stunning and cervical dislocation. Cells were loaded with the cell-permeant acetoxymethyl ester form of indo-1 (2·5 μM for 5 min) left for de-esterification to occur for at least 30 min, and placed in the superfusion chamber on the stage of an inverted microscope adapted for epifluorescence. Indo-1 fluorescence was excited at 340 nm and measured at 400 and 500 nm (O'Neill, Donoso & Eisner, 1990). Voltage-clamp control was achieved using the perforated patch technique (Horn & Marty, 1988). Due to the relatively high access resistance of the perforated patch (about 20 MΩ), the switch clamp facility of the Axoclamp-2A voltage clamp amplifier (Axon Instruments) was used. Pipettes (1–3 MΩ in resistance) were filled with the following solution (mM): 125 $CsCH_3O_3S$, 20 CsCl, 12 NaCl, 10 Hepes and 5 $MgCl_2$; titrated to pH 7·2 with CsOH. To avoid interference from outward currents, all voltage-clamp experiments were carried out in the presence of 5 mM 4-aminopyridine and 0·1 mM $BaCl_2$. Cells were bathed in a control solution of the following composition (mM): 135 NaCl, 4 KCl, 10 Hepes, 11 glucose, 1 $CaCl_2$ and 1 $MgCl_2$; titrated to pH 7·4 with NaOH. All experiments were carried out at 27 °C.

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

In some experiments (Figs 7 and 11), the indo-1 fluorescence records were calibrated in terms of $[Ca^{2+}]_i$ using the equation of Grynkiewicz, Poenie & Tsien (1985), assuming a dissociation constant (K_d) of 220 nM. In these experiments the perforated patch technique was used as above; however, the Ca^{2+} chelator BAPTA (10 mM) was added to the pipette solution (equimolar substitution for $CsCH_3O_3S$). At the end of the experiment the cell membrane was disrupted by applying suction to the electrode obtaining the whole cell configuration, thereby dialysing the cell with BAPTA. The cell was superfused with a Ca^{2+}-free solution. The low ratio obtained under these conditions was taken to be R_{min}. Once a stable R_{min} had been obtained, the superfusate was changed to one containing 5 mM Ca^{2+} and depolarizing pulses were applied to the cell until the ratio reached a steady level. It was verified that this ratio was R_{max} by damaging the cell membrane by lowering the pipette. If this increased the ratio, the higher, final level was taken to be R_{max}. The ratio of the 500 nm signal in Ca^{2+}-free solution to that in high Ca^{2+} solution was taken as β. In ten cells calibrated in this way, the following values were obtained (means ± S.E.M.): R_{max}, 1·5 ± 0·33; R_{min}, 0·4 ± 0·11; β, 2·34 ± 0·10. This method depends on the fact that when the cell is damaged, $[Ca^{2+}]_i$ increases to its maximum value before a significant amount of indo-1 is lost from the cell. If this is not the case, then β will be overestimated. This will lead to an overestimate of $[Ca^{2+}]_i$, however the time courses of changes of $[Ca^{2+}]_i$ will be unaffected.

Measurement of SR Ca^{2+} content and Ca^{2+} efflux

The integrated Na^+–Ca^{2+} exchange current records were converted to total calcium fluxes as described previously (Varro *et al.* 1993; Negretti *et al.* 1995). Briefly, it is necessary to first correct for that fraction of the efflux which is not produced by Na^+–Ca^{2+} exchange and then relate the fluxes to cell volume. This is done by measuring the rate constant of decay of the caffeine response (i) under control conditions (k_{cont}) and (ii) with the Na^+–Ca^{2+} exchange inhibited by 10 mM Ni^{2+} (k_{Ni}). Multiplying the flux by $k_{cont}/(k_{cont} - k_{Ni})$ gives the corrected flux. The volume was calculated from the cell membrane surface area (obtained from the membrane capacitance) and then converted to volume taking a value of 0·5 μm^{-1} for the surface to volume ratio (Page, 1978). It should be noted that, as in our previous work, the SR Ca^{2+} content is expressed with relation to cell (and not SR) volume.

RESULTS

The currents activated by spontaneous oscillations of $[Ca^{2+}]_i$ are illustrated in Fig. 1*A*. The cell was superfused with a solution containing 2 mM calcium and spontaneous oscillations of $[Ca^{2+}]_i$ occurred at a frequency of about 0·2 Hz. The membrane potential was held constant at −80 mV and the transient inward currents activated by each spontaneous oscillation of $[Ca^{2+}]_i$ can be seen in the middle panel. In Fig. 1*B* the same cell was exposed to 10 mM caffeine to release the calcium stored in the SR. The resulting increase of $[Ca^{2+}]_i$ also activates a transient inward current. The currents activated by spontaneous and caffeine-induced release of calcium are thought to result from activation of calcium efflux via Na^+–Ca^{2+} exchange (Fedida *et al.* 1987; Callewaert *et al.* 1989; Varro *et al.* 1993). The calcium lost from the cell during these currents is shown in the bottom panel of each part. Here, the smooth curves represent the integrals of the currents. By relating these integrals to the cell volume (see Methods) we can estimate that, on average in Fig. 1 the Ca^{2+} loss from the cell, on Na^+–Ca^{2+} exchange, per oscillation was 12 μmol l^{-1} and that lost during the application of caffeine was 71 μmol l^{-1}. These values can be corrected for the fact that mechanisms other than Na^+–Ca^{2+} exchange contribute to Ca^{2+} removal from the cytoplasm. Previous work has suggested that Na^+–Ca^{2+} exchange removes 67% of the calcium (Varro *et al.* 1993), although measurements in this study (Table 1) indicate that 80% of efflux is via Na^+–Ca^{2+} exchange. Taking this into consideration, the total Ca^{2+} efflux during the oscillation and caffeine application are 15 and 85 μmol l^{-1}, respectively (all subsequent values have been corrected for the non-Na^+–Ca^{2+} exchange component). Therefore, in this cell, 17% of the SR content is lost during each oscillation. On average, in all twenty-five cells studied, the amount of Ca^{2+} removed from the cell was 15·2 ± 0·5 μmol l^{-1}, corresponding to 13·6 ± 0·7% of the SR Ca^{2+} content.

Effects of external calcium on the magnitude and frequency of spontaneous Ca^{2+} oscillations and SR Ca^{2+} content

The increased frequency of spontaneous oscillations when $[Ca^{2+}]_o$ is raised is illustrated in Fig. 2. Here the cell was oscillating at a low rate in 1 mM bathing calcium. On raising external calcium to 2 mM, resting $[Ca^{2+}]_i$ rose slightly and the frequency of the spontaneous release increased. Raising

Table 1. The effects of external Ca^{2+} concentration on the various components of decay of the caffeine response

External $[Ca^{2+}]$ (mM)	Total	Ni^{2+}	Ni^{2+} sensitive	Fraction of efflux that is Ni^{2+} sensitive
0	1·14 ± 0·03	0·23 ± 0·02	0·91 ± 0·04	0·79 ± 0·02
1	1	0·21 ± 0·03	0·79 ± 0·03	0·79 ± 0·03
5	0·83 ± 0·09	0·17 ± 0·03	0·65 ± 0·07	0·79 ± 0·02

The columns show (from left to right): external Ca^{2+} concentration, rate constant of decay of caffeine response (Total), rate constant of decay in the presence of Ni^{2+} (10 mM), calculated rate constant of decay of the Ni^{2+}-sensitive component, and the fraction of the rate constant that is Ni^{2+} sensitive (i.e. via Na^+–Ca^{2+} exchange). Eight cells were used for the comparison between 0 and 1 mM, and ten cells for that between 1 and 5 mM. All data are normalized to the value in that cell in 1 mM external Ca^{2+}. The mean value of this control rate constant was 0·35 ± 0·04 s^{-1} (n = 18). All cells were held at −80 mV.

the bathing $[Ca^{2+}]_o$ further to 5 mM again increased both resting $[Ca^{2+}]_i$ and the frequency of spontaneous release. It is clear from Fig. 2*A* that the main effect of increased $[Ca^{2+}]_o$ on the oscillations is to increase their frequency with little effect on the magnitude. This is emphasized by the expanded traces of Fig. 2*B*. The right-hand part of Fig. 2*A* shows traces from the same cell but now obtained in the presence of caffeine. This abolishes the spontaneous oscillations and, in addition, changes of $[Ca^{2+}]_o$ now have a much more pronounced effect on resting $[Ca^{2+}]_i$. These effects of caffeine will be considered later.

Experiments such as that illustrated in Fig. 2 demonstrate that elevating $[Ca^{2+}]_o$ produces a marked increase of the frequency of spontaneous SR Ca^{2+} release. In other words the cell appears to be more 'Ca^{2+} overloaded' at higher $[Ca^{2+}]_o$. In subsequent experiments, this has been examined in voltage-clamped cells in order to examine how the Ca^{2+} overload correlates with SR Ca^{2+} content. Figure 3 shows an experiment in which $[Ca^{2+}]_i$ and membrane current were measured in 1, 2 and 5 mM $[Ca^{2+}]_o$. In this cell no oscillations were present in 1 mM $[Ca^{2+}]_o$ and the SR Ca^{2+} content (as judged by the integral of the caffeine-induced current) was

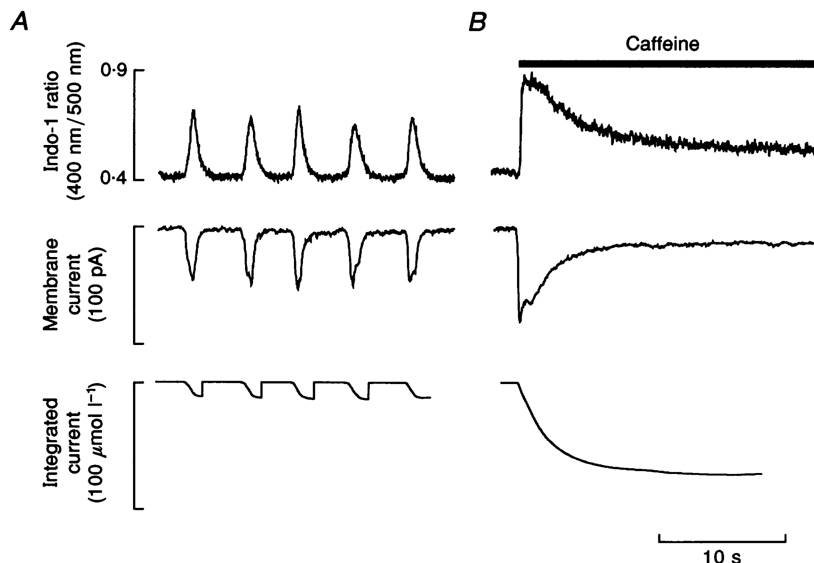

Figure 1. Comparison of spontaneous and caffeine-evoked calcium release

In both *A* and *B*, traces show (from top to bottom): indo-1 ratio (400 nm/500 nm), membrane current and integrated current, converted to total Ca^{2+} movements. In this and subsequent figures, the integrals have been converted to express changes of Ca^{2+} content per cell volume (see Methods). *A*, spontaneous SR Ca^{2+} release. The cell was exposed to 2 mM $[Ca^{2+}]_o$. The membrane potential was held at −80 mV throughout. The integrals have been reset to zero at the end of each current oscillation. *B*, the effects of adding caffeine. Caffeine (10 mM) was applied for the period indicated by the horizontal bar.

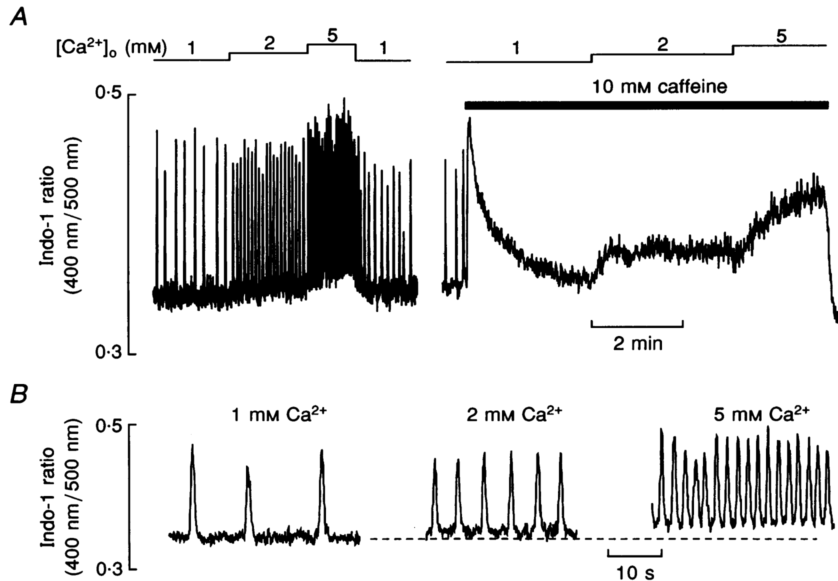

Figure 2. The effects of changing external calcium concentration ($[Ca^{2+}]_o$) on $[Ca^{2+}]_i$

All traces show the indo-1 ratio from an unclamped cell. *A*, time course of changes of $[Ca^{2+}]_i$. $[Ca^{2+}]_o$ was changed between 1, 2 and 5 mM for the periods indicated above the record. In the right-hand panels caffeine (10 mM) was applied for the period shown by the horizontal bar. *B*, expanded records taken from sections in *A* in 1, 2 and 5 mM $[Ca^{2+}]_o$ (in the absence of caffeine).

77 μmol l^{-1}. In 2 mM $[Ca^{2+}]_o$ oscillations appeared at a frequency of about 0·03 Hz. Associated with this was an increase of SR Ca^{2+} content to 106 μmol l^{-1}. A further increase of $[Ca^{2+}]_o$ (to 5 mM) increased the frequency of spontaneous SR Ca^{2+} release, but had no measurable effect on SR Ca^{2+} content. Increasing $[Ca^{2+}]_o$ from 2 to 5 mM slightly decreased the integral of the oscillations of membrane current (from 24 to 17 μmol l^{-1}). On average, however, there was no significant effect of $[Ca^{2+}]_o$ on the integral (see Fig. 5*A*).

The cell illustrated in Fig. 3 was quiescent (i.e. did not show spontaneous oscillations) in the control solution (1 mM $[Ca^{2+}]_o$). This is representative of about 30% of cells (see

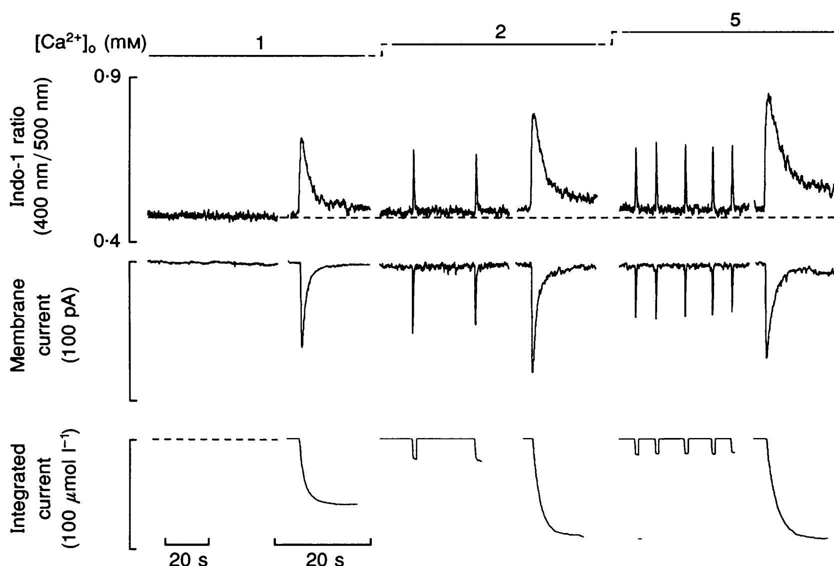

Figure 3. The effects of changing $[Ca^{2+}]_o$ on SR Ca^{2+} content and spontaneous Ca^{2+} release in a cell that did not show spontaneous Ca^{2+} release in 1 mM $[Ca^{2+}]_o$

The traces show (from top to bottom): indo-1 ratio, membrane current and integral of current (for both spontaneous and caffeine-evoked Ca^{2+} release). $[Ca^{2+}]_o$ was changed as shown. The caffeine responses (right-hand traces at each $[Ca^{2+}]_o$) and oscillation records (left-hand traces at each $[Ca^{2+}]_o$) are on different time bases.

Díaz *et al.* 1996). In contrast, Fig. 4 shows data from a cell that was oscillating in 1 mM Ca^{2+}. In this case, elevating $[Ca^{2+}]_o$ to 2 mM markedly increased the rate of these oscillations. This was, however, not accompanied by any change of SR Ca^{2+} content. A further increase of external calcium (to 5 mM) increased the oscillation rate with, again, no effect on SR Ca^{2+} content. These results therefore demonstrate that although over a wide range, an increase of external calcium increases oscillation frequency, the effect on SR Ca^{2+} content is only observed over a more restricted range.

The effects of external calcium on SR Ca^{2+} content and the magnitude and frequency of spontaneous oscillations are shown in Fig. 5*A*; the cells have been separated into two groups: (i) those that were quiescent in 1 mM $[Ca^{2+}]_o$ and (ii) those that were oscillating. In both groups, elevating $[Ca^{2+}]_o$ increased the frequency of the oscillations but had no effect on the amount of Ca^{2+} lost from the cell per oscillation. However, an effect of $[Ca^{2+}]_o$ on SR Ca^{2+} content was only seen over the range of $[Ca^{2+}]_o$ in which the cells were not oscillating. In the non-oscillating cells, on average, increasing $[Ca^{2+}]_o$ from 1 to 2 mM (which resulted in spontaneous SR Ca^{2+} release) increased the SR Ca^{2+} content from 80 ± 6 to 108 ± 13 μmol l^{-1}. A paired *t* test showed that this is a highly significant increase ($P < 0{\cdot}003$, $n = 7$). Furthermore, exposing the cells to a Ca^{2+}-free solution for 1 min decreased the SR Ca^{2+} content by an average of 17 ± 2 μmol l^{-1} ($P < 0{\cdot}002$, $n = 5$). In contrast an increase of $[Ca^{2+}]_o$ from 2 to 5 mM produced no statistically significant change of SR Ca^{2+} content (mean increase of $9{\cdot}1 \pm 15{\cdot}2$ μmol l^{-1}; $P > 0{\cdot}7$, $n = 7$). In the cells that were oscillating in 1 mM $[Ca^{2+}]_o$ (SR Ca^{2+} content 95 μmol l^{-1}), there is no significant change of SR Ca^{2+} content in either 2 or 5 mM (119 μmol l^{-1} in 2 mM and 105 μmol l^{-1} in 5 mM $[Ca^{2+}]_o$; $P > 0{\cdot}09$, $n = 9$). There was, however, a significant decrease of SR Ca^{2+} content, on decreasing external Ca^{2+} to zero (for 1 min) of 29 ± 7 μmol l^{-1} ($P < 0{\cdot}02$, $n = 5$). This was associated with an abolition of spontaneous SR Ca^{2+} release. Taken together, the data show that increasing external Ca^{2+} increases the SR Ca^{2+} content, but only up to a point. This is made more obvious in Fig. 5*B*, which plots oscillation frequency as a function of SR Ca^{2+} content for the cells not oscillating in 1 mM Ca^{2+} and those that were. This shows that the SR Ca^{2+} content can be raised (presumably from 0) to about 90 μmol l^{-1} with no oscillations. However, once this threshold level of SR Ca^{2+} content is passed, then further increase of $[Ca^{2+}]_o$ has no effect on SR Ca^{2+} content, but produces a marked increase of oscillation frequency.

The work described above illustrates that an increase of external calcium increases the loading of the SR. This, in turn, leads to (or allows) spontaneous SR release. In the remainder of this paper two questions are addressed: (i) does the increase of SR Ca^{2+} content result from (a) an increase of Ca^{2+} entry into the cell or (b) a decrease of Ca^{2+} efflux?; (ii) what is the contribution of Ca^{2+} efflux from the cell during spontaneous release to maintenance of cell calcium balance?

The effects of elevated $[Ca^{2+}]_o$ on sarcolemmal Ca^{2+} movement

It is evident from experiments such as that illustrated in Fig. 3 that elevated external calcium tends to slow the decay of the caffeine response. This suggests that increased external calcium lowers the rate of Ca^{2+} removal from the

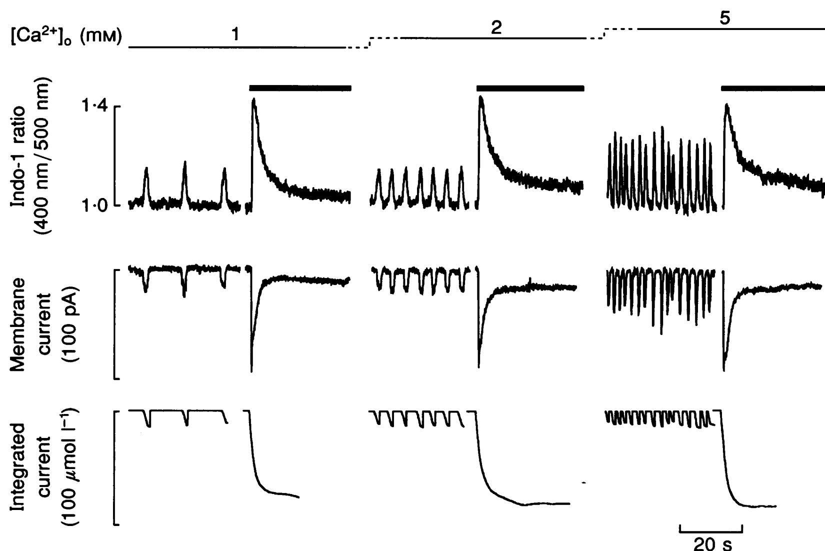

Figure 4. The effects of changing $[Ca^{2+}]_o$ on SR Ca^{2+} content and spontaneous Ca^{2+} release in a cell that showed spontaneous Ca^{2+} release in 1 mM $[Ca^{2+}]_o$

The traces show (from top to bottom): indo-1 ratio, membrane current and integral of current (for both spontaneous and caffeine-evoked Ca^{2+} release). $[Ca^{2+}]_o$ was changed as shown; caffeine application is indicated by horizontal bars.

cytoplasm. This could be due to a decrease in the rate of either Na^+–Ca^{2+} exchange or the Ca^{2+}-ATPase. This has been investigated further by examining the effects of elevated $[Ca^{2+}]_o$ on the rate of decay of the caffeine response, either under control conditions or in the presence of 10 mM Ni^{2+} in order to inhibit the Na^+–Ca^{2+} exchange (Kimura, Miyame & Noma, 1987). The results (Fig. 6 and Table 1) show that increasing external $[Ca^{2+}]$ slows the decay of the caffeine response in both Ni^{2+}-free and Ni^{2+}-containing solutions. The rate constants have been measured in both control and Ni^{2+}-containing solutions and, by subtraction, the total, Ni^{2+}-resistant and Ni^{2+}-sensitive components have been estimated. These are listed in Table 1. The data have been normalized to the value of the total rate constant in 1 mM $[Ca^{2+}]_o$. Elevating $[Ca^{2+}]_o$ from 1 to 5 mM decreases the total flux by about 20%. However, the fraction of the efflux that is Ni^{2+} sensitive remains constant at 79%. These data show that raising external calcium inhibits the Ca^{2+} efflux by both Na^+–Ca^{2+} exchange and Na^+–Ca^{2+} exchange-independent routes, more or less equally.

The effects of external calcium on Na^+–Ca^{2+} exchange can be studied in another way by examining the relationship between Na^+–Ca^{2+} exchange current and $[Ca^{2+}]_i$ during a caffeine response. Figure 7*A* shows caffeine responses in two different external calcium concentrations. The relationship between current and $[Ca^{2+}]_i$ shows a marked hysteresis (Trafford, Díaz, O'Neill & Eisner, 1995). Previous work suggests that the relationship approximates to the steady state during the decay of $[Ca^{2+}]_i$ and this relationship is shown by the continuous lines in Fig. 7*B*. It is clear that the slope is decreased in elevated $[Ca^{2+}]_o$. On average, increasing external Ca^{2+} from 1 to 5 mM decreased the slope to 0.63 ± 0.16 ($n = 5$) of the control level. This can be compared with the effects of elevating $[Ca^{2+}]_o$ on the Ni^{2+}-sensitive (i.e. Na^+–Ca^{2+} exchange) component of the rate constant of decay of the caffeine response (Fig. 6). The average value of (Ni^{2+}-sensitive rate constant in 5 mM $[Ca^{2+}]_o$)/(Ni^{2+}-sensitive rate constant in 1 mM $[Ca^{2+}]_o$) was 0.74 ± 0.14 ($n = 8$). This is not significantly different from the effect on the slope (Student's unpaired t test, $P > 0.2$). Decreasing $[Ca^{2+}]_o$ to 0 mM increased the slope to $122 \pm 4\%$ ($n = 4$).

As there is no obvious direct way to measure the rate of Ca^{2+} entry into the cell, it is assumed that, in the steady state, the Ca^{2+} entry into the cell is exactly balanced by Ca^{2+} efflux. Therefore if the Ca^{2+} efflux can be measured, the entry

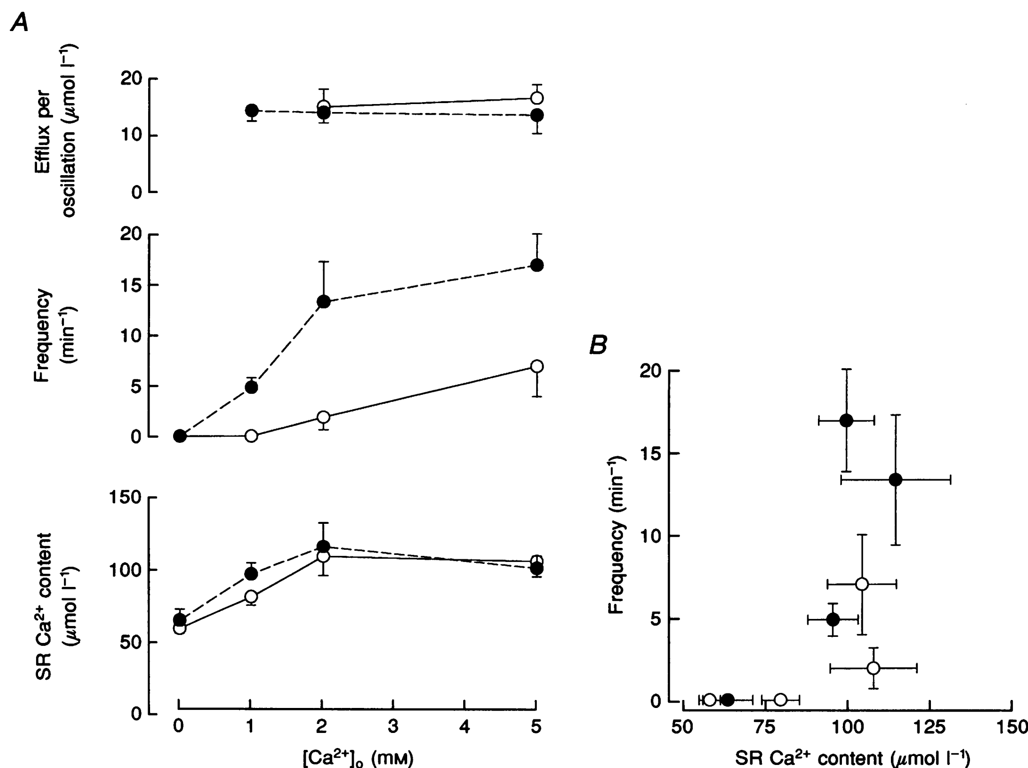

Figure 5. Summary of the effects of $[Ca^{2+}]_o$ on SR Ca^{2+} content and spontaneous SR Ca^{2+} release

A, effect of external calcium concentration on (from top to bottom): calculated Ca^{2+} efflux per oscillation, frequency of spontaneous release and SR Ca^{2+} content. The open symbols (○) are from cells that did not show spontaneous SR release in control solution (1 mM $[Ca^{2+}]_o$). The filled symbols (●) are from cells that were oscillating in 1 mM $[Ca^{2+}]_o$. The cells were exposed to the test solutions for 1 min before the measurements were made. *B*, the relationship between SR Ca^{2+} content (abscissa) and frequency of spontaneous SR Ca^{2+} release (ordinate). The filled and open symbols represent the same data as in *A*. For each symbol, the four points represent 0, 1, 2 and 5 mM $[Ca^{2+}]_o$. All cells were held at −80 mV.

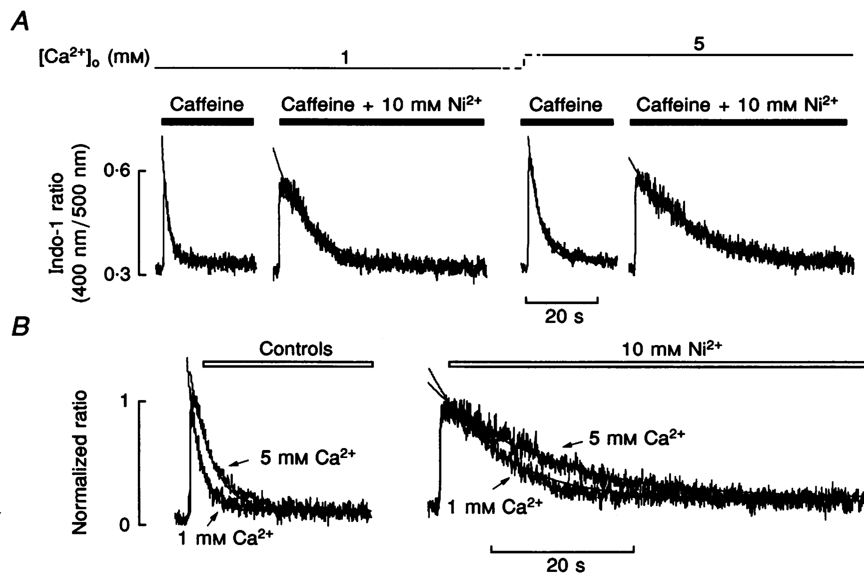

Figure 6. The effects of $[Ca^{2+}]_o$ on the decay of the caffeine response

A, original records of the effects of adding caffeine (10 mM) on $[Ca^{2+}]_i$. The records were obtained in 1 and 5 mM $[Ca^{2+}]_o$ as indicated. Membrane potential was held at −80 mV. At each $[Ca^{2+}]_o$, caffeine was applied both in the absence and presence of Ni^{2+} (10 mM). Single exponential fits are shown with the following rate constants (from left to right): 0·60, 0·11, 0·32 and 0·06 s^{-1}. *B*, normalized traces. The records on the left were obtained in the absence of Ni^{2+} and those on the right in the presence of Ni^{2+}.

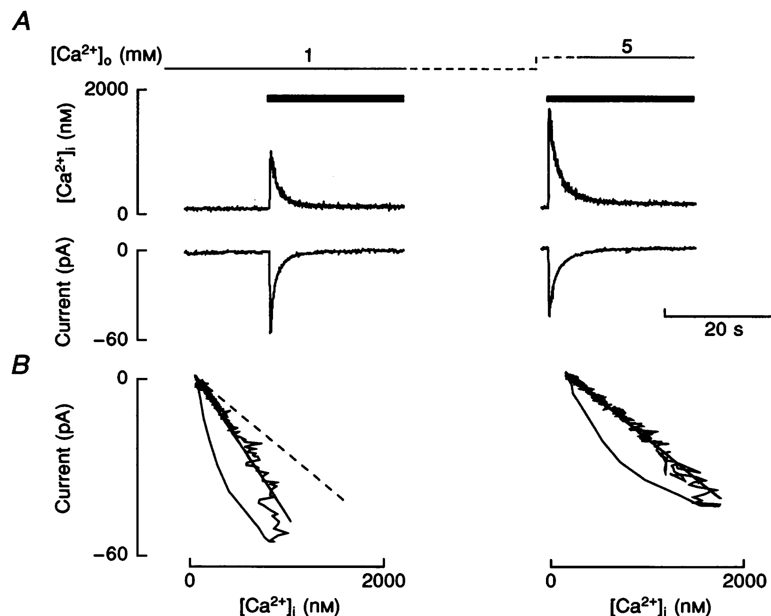

Figure 7. The effects of external Ca^{2+} concentration on the relationship between Na^+–Ca^{2+} exchange current and $[Ca^{2+}]_i$

A, original records. Both panels show the effects of applying caffeine (10 mM; indicated by horizontal bars). In the right-hand panel external Ca^{2+} had been elevated from 1 to 5 mM. *B*, the relationship between current and $[Ca^{2+}]_i$. A linear regression has been drawn through the phase of decreasing $[Ca^{2+}]_i$ and current. In the left-hand panel (1 mM $[Ca^{2+}]_o$), the dashed line shows the linear regression from the 5 mM $[Ca^{2+}]_o$.

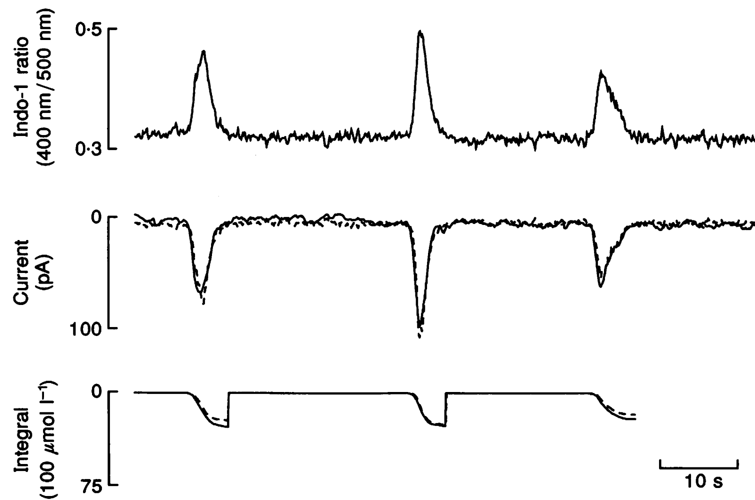

Figure 8. Test of the method for estimating Ca^{2+} efflux

Traces show (from top to bottom): indo-1 ratio, membrane current and integral. The membrane potential was held at −80 mV. These were obtained in 2 mM $[Ca^{2+}]_o$. For both the membrane current and the calculated integral two traces are shown. The continuous lines show the original current record and integral. The dashed lines were calculated from the indo-1 ratio using the relationship obtained from the caffeine response in 1 mM $[Ca^{2+}]_o$.

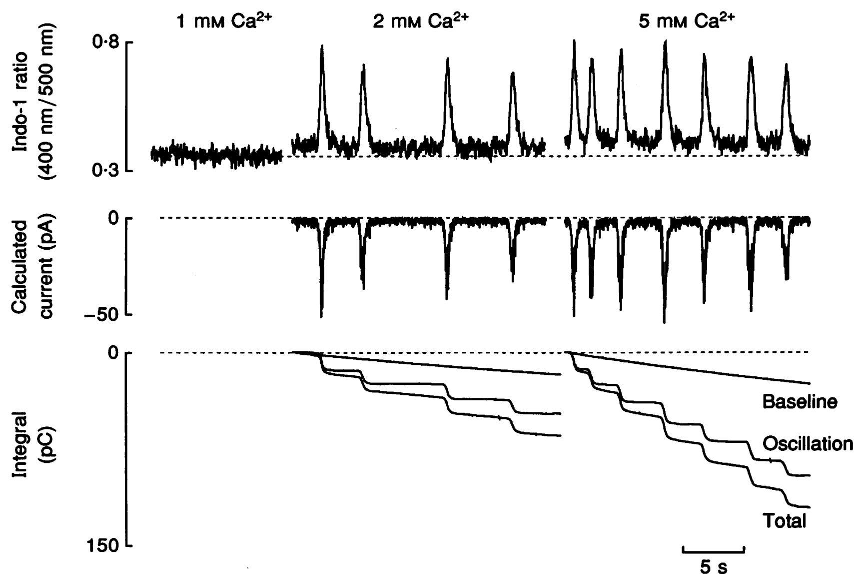

Figure 9. Calculation of the contribution of spontaneous SR Ca^{2+} release and elevated resting $[Ca^{2+}]_i$ to the extra Ca^{2+} efflux produced by elevating $[Ca^{2+}]_o$

The membrane potential was held at −80 mV. Traces show (from top to bottom): indo-1 ratio, calculated current and calculated integrals. The calculated currents were obtained from the indo-1 ratios shown in the top traces and the relationship between indo-1 ratio and current (not shown). The zero level for this calculated current is set such that the current in 1 mM $[Ca^{2+}]_o$ is zero. In the bottom panel three integral traces are shown. The total is the cumulative integral of the calculated current. The other two integral records show this broken down into the contributions from spontaneous Ca^{2+} release (obtained by integrating only the oscillations) and the increase of baseline Ca^{2+}.

can be estimated. Experiments such as those illustrated in Fig. 2 show that the effect of elevating external Ca^{2+} is to produce (i) an increase of resting $[Ca^{2+}]_i$ and (ii) spontaneous oscillations of $[Ca^{2+}]_i$. Both of these will tend to increase Ca^{2+} efflux by activating the Ca^{2+} removal processes. The aim of this section of the paper is to estimate the relative contribution of these two mechanisms to Ca^{2+} efflux and thence to the control of cell Ca^{2+} content. The amount of Ca^{2+} that is pumped out of the cell during each oscillation can be measured by directly integrating the Na^+–Ca^{2+} exchange current. It is, however, not possible to measure the fluxes activated by the rise of resting $[Ca^{2+}]_i$, as raising $[Ca^{2+}]_i$ by this means is associated with an outward current, perhaps due to an external effect of raised $[Ca^{2+}]_o$. Instead, the following approach has been adopted.

Estimation of Ca^{2+} efflux due to elevated resting $[Ca^{2+}]_i$

The relationship between Na^+–Ca^{2+} exchange current and $[Ca^{2+}]_i$ is obtained during the falling phase of the caffeine response (as in Fig. 7). Once this is obtained, then provided that only $[Ca^{2+}]_i$ has changed, the Na^+–Ca^{2+} exchange current can be estimated from a measurement of $[Ca^{2+}]_i$. The validity of this technique can be tested by comparing the calculated Na^+–Ca^{2+} exchange current measured during an oscillation with that measured directly, as in Fig. 8. The top trace shows three spontaneous oscillations of $[Ca^{2+}]_i$. The continuous line in the middle trace shows the measured currents associated with these oscillations. The current as calculated from the indo-1 ratio and the relation between $[Ca^{2+}]_i$ and current is shown by the dashed line. The calculated current is similar in size and time course to the measured current. The bottom trace shows that the integrals of the measured and calculated currents are also similar in size. On average, in 2 mM $[Ca^{2+}]_o$ the calculated integral was 94 ± 7% ($n = 6$) and in 5 mM $[Ca^{2+}]_o$ was 115 ± 10% ($n = 6$) of the measured integrals. Neither of these is significantly different from 100% (paired t test; $P > 0{\cdot}4$ and $0{\cdot}2$, respectively). In some experiments (e.g. Figs 8 and 9) the cell was not calibrated in terms of absolute units of $[Ca^{2+}]_i$. In those cases a general non-linear curve is fitted to the relationship between indo-1 ratio and current. This alternative technique did not affect the excellent agreement between calculated and measured current.

This procedure has been used to estimate the fluxes of calcium activated by spontaneous oscillations and by the maintained elevation of $[Ca^{2+}]_i$. The top trace in Fig. 9 shows $[Ca^{2+}]_i$ in 1, 2 and 5 mM $[Ca^{2+}]_o$. The dashed line is drawn through the level of $[Ca^{2+}]_i$ in 1 mM $[Ca^{2+}]_o$. This was used to calculate the basal level of Ca^{2+} efflux. The middle trace shows the calculated membrane current with respect to this basal level. The zero current level represents the level of current calculated to be flowing at the resting $[Ca^{2+}]_i$ in 1 mM external calcium, i.e. any downward deflection of current represents extra efflux of calcium on Na^+–Ca^{2+} exchange above that present in 1 mM external calcium. In the lower panel the smooth curve shows the integral of the current calculated to flow as a result of the increase in resting $[Ca^{2+}]_i$. The middle stepping trace shows the integral of the current due only to efflux during spontaneous release and the bottom trace represents the total efflux during the

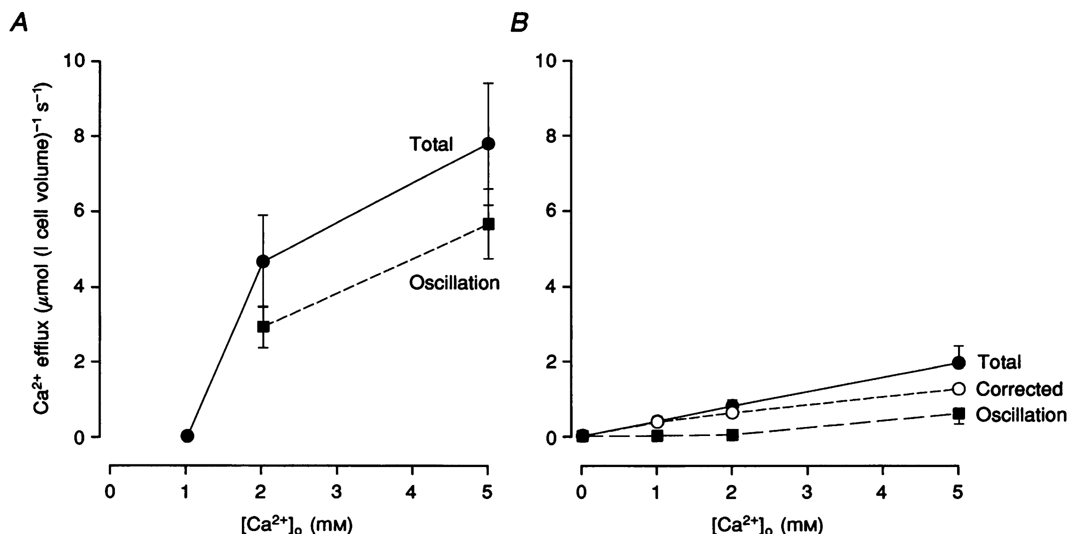

Figure 10. The relationship between external Ca^{2+} concentration and Ca^{2+} influx

A, cells oscillating in 1 mM $[Ca^{2+}]_o$. Here all fluxes are relative to 0 mM $[Ca^{2+}]_o$. ●, total Ca^{2+} efflux; ■, flux through the oscillations. These were calculated as shown for Fig. 9. *B*, cells not oscillating in 1 mM $[Ca^{2+}]_o$. Here the measurements are with respect to 1 mM $[Ca^{2+}]_o$. ○, flux corrected for the effects of external calcium on the Ca^{2+} removal mechanisms. These points were obtained by multiplying the total flux by the ratio of the rate constant of decay of the caffeine response in the test external Ca^{2+} concentration divided by that in 1 mM Ca^{2+}.

period shown. It is obvious that the total Ca^{2+} loss has a steeper gradient in 5 mM than in 2 mM $[Ca^{2+}]_o$. In this experiment the fraction of the Ca^{2+} loss activated by spontaneous SR Ca^{2+} release was 80% of the total in both 2 and 5 mM $[Ca^{2+}]_o$. On average, the fraction of the extra efflux on elevating $[Ca^{2+}]_o$ that occurred during the oscillations was $55{\cdot}4 \pm 5{\cdot}8$% in 2 mM and $60{\cdot}7 \pm 4{\cdot}5$% in 5 mM Ca^{2+} ($n = 9$).

Estimation of total Ca^{2+} fluxes

The method described above was used to calculate the relative contribution of elevated baseline and oscillations to the elevated calcium efflux produced by increasing $[Ca^{2+}]_o$ from its initial level of 1 mM. It can also be used to measure the total efflux of calcium from the cell and thence the influx (assuming influx equals efflux). Figure 10 again shows this separated by whether or not the cell was oscillating. The cells illustrated in Fig. 10*A* were oscillating in 1 mM $[Ca^{2+}]_o$. The fluxes are shown with respect to the level in 1 mM $[Ca^{2+}]_o$. It is obvious that the flux increases with elevated $[Ca^{2+}]_o$. The dashed line shows the fraction of the efflux that is due to oscillations. In these cells this is a large fraction. Figure 10*B* shows similar data from cells which were not oscillating in 1 mM $[Ca^{2+}]_o$. Again the Ca^{2+} flux increases with $[Ca^{2+}]_o$, but the magnitude of the slope is now considerably less. In this figure, the fluxes are shown with respect to the level in Ca^{2+}-free solution, therefore allowing an estimate of the total Ca^{2+} influx, rather than simply the increment over the level in 1 mM $[Ca^{2+}]_o$.

It should be noted that these calculations estimate Ca^{2+} at several values of $[Ca^{2+}]_o$ from the relationship between Ca^{2+} and current obtained at a single $[Ca^{2+}]_o$. However, as shown above, the slope of the relationship between $[Ca^{2+}]_i$ and Na^+-Ca^{2+} exchange current decreases as $[Ca^{2+}]_o$ is increased and this must be allowed for. The open symbols in Fig. 10*B* show the data corrected for this. This correction does not affect the fractional contribution of spontaneous release to efflux.

Control of $[Ca^{2+}]_i$ in the absence of spontaneous SR Ca^{2+} release

It has already been seen (Fig. 2) that under control conditions, elevating $[Ca^{2+}]_o$ produces only a small increase of resting $[Ca^{2+}]_i$. However, in the presence of caffeine, along with the abolition of oscillations there is a marked elevation of $[Ca^{2+}]_i$ on increasing $[Ca^{2+}]_o$. The experiment illustrated in Fig. 11 shows the effects of adding caffeine at different calcium concentrations. In the nominal absence of external calcium the response to caffeine is almost entirely transient. Any small maintained component may be due to problems of ratioing the two wavelengths in the presence of the quench produced by caffeine (O'Neill *et al.* 1990). In 5 mM $[Ca^{2+}]_o$, however, there is an appreciable maintained component in caffeine. There was no spontaneous Ca^{2+} release in the Ca^{2+}-free solution. However, spontaneous release is evident in 5 mM $[Ca^{2+}]_o$. Therefore the elevated maintained $[Ca^{2+}]_i$ in the presence of caffeine is seen when the cell was previously oscillating. The maintained component is greater than the diastolic level of the previous Ca^{2+} oscillations, but lower than their peak level. Using the method described above, one can calculate the Ca^{2+} efflux in the absence and presence of oscillations. These have been calculated with respect to the levels in the absence of external calcium. In this cell the extra Ca^{2+} efflux was $4{\cdot}1\ \mu mol\ l^{-1}\ s^{-1}$ in 1 mM $[Ca^{2+}]_o$ in the absence of caffeine (i.e. the sum of the effects of elevated baseline plus oscillations) and $5{\cdot}9\ \mu mol\ l^{-1}\ s^{-1}$ in the presence of caffeine. In seven cells, the extra Ca^{2+} efflux in 5 mM $[Ca^{2+}]_o$ above that in Ca^{2+}-free solution has been measured. On average this was

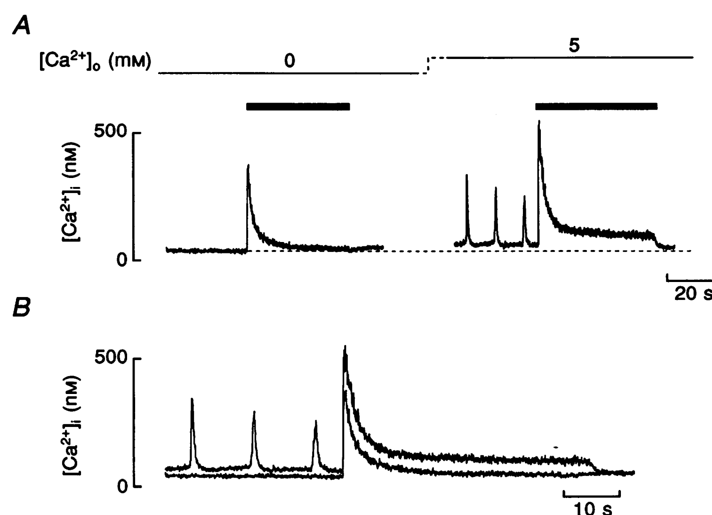

Figure 11. The effects of external calcium concentration on the steady-state change of $[Ca^{2+}]_i$ produced by caffeine (membrane potential, −80 mV)

A, the effects of adding caffeine (10 mM; indicated by horizontal bars) in either 0 or 5 mM $[Ca^{2+}]_o$. *B*, the responses are shown superimposed.

$3 \cdot 3 \pm 0 \cdot 84$ μmol l^{-1} s^{-1} in the absence of caffeine (while the cell was oscillating) and $3 \cdot 8 \pm 1 \cdot 1$ μmol l^{-1} s^{-1} in the presence of caffeine. The flux in the presence of caffeine was, on average, $1 \cdot 2 \pm 0 \cdot 1$ of that in the absence of caffeine. The ratio is not significantly different from 1·0 ($P > 0 \cdot 7$; paired t test). In other words, the addition of caffeine has no effect on the calculated flux across the surface membrane, but that component of efflux which would previously have been activated by oscillations is, in the presence of caffeine, activated by an elevated baseline.

DISCUSSION

Both SR and surface membrane function have been studied during the development of calcium overload produced by elevating external calcium concentration. The results show that spontaneous calcium release limits the SR Ca^{2+} content to a maximum level at which release occurs. Furthermore, the calcium removed from the cell during spontaneous release makes a significant contribution to cellular calcium homeostasis.

The effects of elevating external calcium on spontaneous SR Ca^{2+} release and Ca^{2+} content

Increasing external Ca^{2+} concentration increases the frequency of spontaneous SR Ca^{2+} release (Capogrossi, Stern, Spurgeon & Lakatta, 1988). Over the range of $[Ca^{2+}]_o$ studied here, the relationship is monotonic and shows no obvious tendency towards saturation. The amount of Ca^{2+} pumped out of the cell per oscillation does not change with external Ca^{2+} concentration or the degree of Ca^{2+} overload. The increase of Ca^{2+} efflux from the cell with increased 'Ca^{2+} overload' is therefore due to increasing frequency rather than magnitude of oscillation-induced Ca^{2+} efflux. Given that the amount of Ca^{2+} pumped out of the cell is constant, it is likely that the amount released from the SR is also constant. Indeed, as shown in previous work on cardiac cells (Capogrossi *et al.* 1988), the magnitude of the oscillatory increase of $[Ca^{2+}]_i$ does not appear to increase with increasing degree of Ca^{2+} overload. Similarly, in bullfrog sympathetic neurones, increasing Ca^{2+} load affects the frequency but not the amplitude or shape of the Ca^{2+} transients produced by spontaneous release (Friel & Tsien, 1992).

The relationship between Ca^{2+} load and SR Ca^{2+} content is rather more complicated. In cells that were not oscillating in the control (1 mM) $[Ca^{2+}]_o$, when external Ca^{2+} was elevated to 2 mM, resulting in spontaneous SR Ca^{2+} release, then the SR Ca^{2+} content increased. However, further increase of $[Ca^{2+}]_o$, although increasing the oscillation frequency, had no significant effect on SR Ca^{2+} content. Thus SR Ca^{2+} content only changes over the range of calcium in which no spontaneous SR Ca^{2+} release is present. This is also consistent with the fact that, in cells that show spontaneous Ca^{2+} release in 1 mM $[Ca^{2+}]_o$, increase of $[Ca^{2+}]_o$, despite increasing the frequency of spontaneous SR release, has no effect on SR Ca^{2+} content. In all cells, reduction of $[Ca^{2+}]_o$ to zero decreased SR Ca^{2+} content. These results should be compared with previous work, which used the magnitude of the rapid cooling contraction to obtain a qualitative estimate of SR Ca^{2+} content (Bers & Bridge, 1988) where it was found that the application of acetylstrophanthidin first increased the SR Ca^{2+}; there was no further increase at concentrations that produced calcium overload. Previous work has suggested that spontaneous Ca^{2+} release may limit inotropic manoeuvres either by depleting the SR (Allen, Eisner, Pirolo & Smith, 1985) and/or by inactivating Ca^{2+} release (Capogrossi *et al.* 1988). This paper shows, directly, that the spontaneous Ca^{2+} release indeed limits the SR Ca^{2+} content.

The accuracy of the measurements of SR Ca^{2+} content from the integrals of the caffeine-evoked current should be considered. This has been discussed previously (Varro *et al.* 1993; Negretti *et al.* 1995). However, in the present work it is important to consider the effects of changes of external calcium. One concern is that an increase of $[Ca^{2+}]_o$ could inhibit the Na^+–Ca^{2+} exchange more than the surface membrane Ca^{2+}-ATPase. This would mean that, as $[Ca^{2+}]_o$ was elevated, the Na^+–Ca^{2+} exchange would underestimate the total Ca^{2+} efflux. Similarly, if the Ca^{2+}-ATPase was more sensitive than the Na^+–Ca^{2+} exchange to external Ca^{2+} then the Na^+–Ca^{2+} exchange current would overestimate the Ca^{2+} efflux. The results (Table 1) show, however, that both processes are similarly inhibited by elevating external Ca^{2+}.

The overall relationship between SR Ca^{2+} content and frequency of spontaneous SR Ca^{2+} release (Fig. 5*B*) shows that, up to an SR Ca^{2+} content of about 90 μmol l^{-1}, there is no spontaneous SR Ca^{2+} release. Above this level, however, spontaneous release develops and further increase of $[Ca^{2+}]_o$ has no effect on SR Ca^{2+} content. One explanation for this result is provided if we assume that spontaneous Ca^{2+} release from the SR occurs when the SR Ca^{2+} content reaches a certain critical level (Fabiato, 1983). As long as the SR Ca^{2+} content is below this level then an increase of $[Ca^{2+}]_o$ and therefore (see later) of Ca^{2+} entry into the cell will increase the SR Ca^{2+} load. However, once the critical SR Ca^{2+} content is reached, then spontaneous Ca^{2+} release will occur, as a result of which some of the released Ca^{2+} will be pumped out of the cell. The SR will then refill as Ca^{2+} enters the cell. The greater the rate of Ca^{2+} entry into the cell, the less time it will take for the SR to refill to the critical level and therefore the higher the frequency of spontaneous Ca^{2+} release.

This suggests that the content of the SR may somehow determine when spontaneous release takes place. This may arise in two ways: (i) an increase of the luminal SR calcium content may directly increase the open probability of the ryanodine receptor (Sitsapesan & Williams, 1994) or (ii) when SR Ca^{2+} content is increased, a given opening probability will produce a greater flux of calcium and therefore any local spontaneous release event e.g. a spark (Cheng, Lederer & Cannell, 1993; López-López, Shacklock, Balke & Wier, 1994) will be able to initiate a propagating wave of calcium release (Trafford, O'Neill & Eisner, 1993; Han, Schiefer &

Isenberg, 1994; Bassani, Yuan & Bers, 1995). In the experiments of this study, it is not possible to distinguish between these effects.

The hypothesis that, irrespective of the rate of Ca^{2+} loading of the cell, the SR Ca^{2+} content just before an oscillation has the same critical level, is supported by the fact that the magnitude of the Ca^{2+} oscillation is reasonably constant. It is difficult to measure this directly from the Ca^{2+} records; the apparent peak magnitude of the Ca^{2+} records depends not only on the magnitude of the Ca^{2+} wave at any point in the cell but, in addition, on the speed of propagation of the wave and thence on the degree of spatial uniformity of the wave (Takamatsu & Wier, 1990). A better estimate is given by the integral of the Na^+–Ca^{2+} exchange current, which varies very little over a wide range of frequency of Ca^{2+} release. It is also worth noting that the present experiments were performed on rat myocytes, which show spontaneous release more readily than do cells from other species (Kort & Lakatta, 1988). However, when the Ca^{2+} load is increased even other species show spontaneous release and one would expect the present results to be generally applicable.

In conclusion, the results of this section suggest that once 'calcium overload' (as defined by the presence of spontaneous SR calcium release) is reached, then even if the cell becomes more 'overloaded' (as shown by an increased frequency of spontaneous release) the SR Ca^{2+} content does not increase further. Calcium overload is therefore more a measure of fluxes across the surface membrane than of cell calcium content.

The effects of external Ca^{2+} on Ca^{2+} efflux and entry

The experiments show (Fig. 6) that elevating external Ca^{2+} lowers the rate of Ca^{2+} removal from the cell. This was studied initially by measuring the rate of decline of the caffeine-evoked increase of $[Ca^{2+}]_i$. Raising $[Ca^{2+}]_o$ from 1 to 5 mM decreased the overall rate constant to 83%. There were similar effects on the Na^+–Ca^{2+} exchange (Ni^{2+} sensitive) and the non-Na^+–Ca^{2+} exchange processes. A more direct measure of the effects of elevated $[Ca^{2+}]_o$ on the Na^+–Ca^{2+} exchange component comes from studying the slope of the relationship between current and $[Ca^{2+}]_i$ during the decay of the caffeine response, which was also decreased in 5 mM compared with 1 mM $[Ca^{2+}]_o$.

An increase in external calcium concentration will produce an increased rate of Ca^{2+} entry into the cell. This, in turn, must be balanced by an increase of Ca^{2+} efflux if the cell is to be able to regulate $[Ca^{2+}]_i$. Unless the properties of the Ca^{2+} removal processes are changed, such an increased Ca^{2+} efflux will require an increase in time-averaged $[Ca^{2+}]_i$. This is brought about in two ways: (i) an increase of oscillation frequency or (ii) an increase of resting $[Ca^{2+}]_i$. The rate of Ca^{2+} efflux from the cell during oscillations could be measured directly from the Na^+–Ca^{2+} exchange current. On average, the Ca^{2+} loss from the cell during each oscillation was $15 \cdot 2 \pm 0 \cdot 5$ μmol l^{-1}. This was unaffected by the frequency of Ca^{2+} oscillations. Results such as that in Fig. 2 show that Ca^{2+} oscillations can occur with very little rise of resting $[Ca^{2+}]_i$, suggesting that the loss during oscillations may be very important. In order to make a quantitative comparison, one requires an estimate of the Ca^{2+} efflux produced by the increase of resting $[Ca^{2+}]_i$. This was done by measuring the relationship between Na^+–Ca^{2+} exchange current and $[Ca^{2+}]_i$ during the decay of a caffeine response and applying this to the elevation of $[Ca^{2+}]_i$ in order to estimate the Ca^{2+} efflux. This assumes, of course, that the only factor that affects Na^+–Ca^{2+} exchange current is the increase of $[Ca^{2+}]_i$, and that the level of $[Ca^{2+}]_i$ 'seen' by the Na^+–Ca^{2+} exchange is the same as that measured by the fluorescent indicator. The latter assumption is not entirely correct, as there is evidence that, during spontaneous SR Ca^{2+} release, subsarcolemmal and bulk $[Ca^{2+}]_i$ are not identical (Lipp, Pott, Callewaert & Carmeliet, 1990; Trafford *et al.* 1995). However, the experimental results show (Fig. 8) that, when a comparison can be made, the calculated integrated current agrees with the measured one. This may be because during SR Ca^{2+} release, subsarcolemmal Ca^{2+} is greater than the bulk concentration, whereas during reuptake it is less; averaged over the whole cycle these effects may therefore cancel out. Another potential problem is that, as mentioned above, external calcium decreases the activity of the exchange. The effect of this (a 20% reduction on increasing external calcium from 1 to 5 mM) is that the calculated calcium efflux is 20% greater than actually takes place. One would therefore expect that the estimated Ca^{2+} efflux during an oscillation in 5 mM Ca^{2+} would be 20% greater than that actually measured. The observed value of the ratio of estimated/measured integrals is 115 ± 10%, which is not significantly different from the expected value of 120%. However, it is equally not significantly different from 100%, and the data are not therefore sufficiently precise to show the expected effect of inhibition by external calcium. The conclusion, then, is that the method of estimating Ca^{2+} efflux is reliable. However, in some cases (Fig. 10) the effects of external calcium on the Ca^{2+} efflux pathways have been corrected for. It is important to note, however, that uncertainties in this correction will not affect the estimate of the fraction of calcium efflux that occurs via Ca^{2+} oscillations, as opposed to via the increase of resting $[Ca^{2+}]_i$.

It is clear from the results that the loss of Ca^{2+} during oscillations is a significant fraction (50–60%) of the total loss during overload. One consequence of this is that, when spontaneous Ca^{2+} release is inhibited, there is a marked increase of resting $[Ca^{2+}]_i$ (Figs 2 and 11). The data show that, under a given set of conditions, the Ca^{2+} efflux from the cell is the same no matter whether the cell is undergoing spontaneous release or if this release has been inhibited with caffeine. This is consistent with the hypothesis that, in the steady-state, Ca^{2+} efflux must balance the Ca^{2+} entry. Under Ca^{2+}-overloaded conditions this is achieved by a combination of spontaneous Ca^{2+} release and elevated diastolic $[Ca^{2+}]_i$. If, however, the spontaneous release is inhibited then the diastolic $[Ca^{2+}]_i$ must be elevated until

the same level of Ca^{2+} efflux is achieved. These results are somewhat different from those found previously on aequorin-injected cells from Purkinje fibres where abolition of oscillations appeared to decrease mean $[Ca^{2+}]_i$ (Eisner & Valdeolmillos, 1986). However, it is possible that spatial non-uniformities in $[Ca^{2+}]_i$ combined with the supra-linear relationship between $[Ca^{2+}]_i$ and aequorin light could have led to an overestimate of time-averaged $[Ca^{2+}]_i$ in the presence of spontaneous SR release.

Can spontaneous Ca^{2+} release have a beneficial effect? Much attention has been paid to the unwanted effects of spontaneous SR Ca^{2+} release and, in particular, to the production of afterdepolarizations and thence some cardiac arrhythmias (Ferrier, Saunders & Mendez, 1973; Kass, Lederer, Tsien & Weingart, 1978). However, the results of this paper show that under Ca^{2+}-overloaded conditions, the Ca^{2+} loss through spontaneous Ca^{2+} release is an alternative to that reached through an increase of resting $[Ca^{2+}]_i$. Although the former may result in cardiac arrhythmias, the latter will cause diastolic tension development and thus impair cardiac filling. At moderate levels of Ca^{2+} overload (low frequencies of spontaneous SR Ca^{2+} release), the spontaneous release will be out of phase between different cells and the resulting depolarizations may be too small to excite an action potential. Similarly, asynchronous oscillatory release will generate less tension than a maintained rise of $[Ca^{2+}]_i$ that produced the same calcium efflux. It is therefore possible that spontaneous release is teleologically 'preferable' to elevated diastolic $[Ca^{2+}]_i$.

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