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BUFFERING OF CALCIUM INFLUX BY SARCOPLASMIC RETICULUM DURING THE ACTION POTENTIAL IN GUINEA-PIG VENTRICULAR MYOCYTES

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SUMMARY

1. Intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) transients, monitored by the fluorescent Ca^{2+} indicator, indo-1, and twitch contractions elicited by action potentials, by voltage clamp pulses or by rapid, brief pulses of caffeine, were measured in guinea-pig single ventricular myocytes. Experiments were designed to determine whether and to what extent the trans-sarcolemmal Ca^{2+} influx is immediately sequestered by the sarcoplasmic reticulum (SR).

2. Rapid, brief (100–200 ms) pulses of caffeine onto a rested myocyte elicited a $[Ca^{2+}]_i$ transient and a contraction. Following exposure to specific SR inhibitors, ryanodine (100 nM) or thapsigargin (200 nM), the rapid application of caffeine onto a rested myocyte failed to elicit changes in $[Ca^{2+}]_i$ or in cell length, indicating that caffeine increases $[Ca^{2+}]_i$ by specifically discharging Ca^{2+} from the SR. In the absence of these inhibitors, a second pulse of caffeine, within 3 min following a prior pulse, failed to elicit a $[Ca^{2+}]_i$ transient or contraction, indicating that a caffeine pulse depletes the SR releasable Ca^{2+} pool.

3. Following Ca^{2+} depletion of the SR by double caffeine pulses at rest, an electrical stimulation elicited a slow increase in $[Ca^{2+}]_i$, and, after a delay, a small, slow twitch contraction. The simultaneous application of caffeine and electrical stimulation of cells in which the SR was Ca^{2+} depleted elicited $[Ca^{2+}]_i$ transients with an increased rate of rise and a larger amplitude (53 ± 8 and 63 ± 9% respectively; mean $\pm$ S.E.M., $n = 21$) than those elicited by electrical stimulation alone.

4. Whether caffeine affected the L-type calcium current (I_{Ca}) elicited by electrical stimulation was determined under whole-cell voltage clamp. A caffeine pulse delivered at the onset of a depolarizing voltage clamp step also increased the rates of rise and the amplitudes of the $[Ca^{2+}]_i$ transients and twitch contractions in cells in which the SR was depleted of Ca^{2+}. However, Ca^{2+} influx via I_{Ca} decreased when caffeine was pulsed in conjunction with the voltage clamp, as the peak I_{Ca} was either unchanged or decreased while its inactivation was consistently accelerated.

5. Because the stimulation-dependent trans-sarcolemmal Ca^{2+} influx via I_{Ca} is not increased by a caffeine pulse, the augmentation of the rates of rise and the amplitudes of the electrically stimulated $[Ca^{2+}]_i$ transients by caffeine pulsed in conjunction with

the electrical stimulation in cells in which the SR had been depleted of Ca^{2+} indicates that a portion of Ca^{2+} influx during depolarization in the absence of caffeine is rapidly buffered by the SR. Accordingly, the selective inhibition of the SR Ca^{2+} uptake by thapsigargin also increased the rates of rise and the amplitudes of the post-rest $[Ca^{2+}]_i$ transients elicited by electrical stimulation by 65 ± 21 and 28 ± 8%, respectively. The rates of rise and the amplitudes of the accompanying twitch contractions were increased by 57 ± 21 and 112 ± 27%, respectively ($n = 7$).

6. The caffeine-dependent augmentation of the electrically stimulated $[Ca^{2+}]_i$ transients in cells in which the SR had been depleted of Ca^{2+} decreased if the caffeine pulse was delivered after a delay following electrical stimulation. This suggests that the rapid sequestration of the transmembrane Ca^{2+} influx by the SR during electrical stimulation is followed by SR Ca^{2+} release during the same depolarization.

7. We conclude that during a single post-rest stimulation of guinea-pig ventricular myocytes, at least 50% of Ca^{2+} entering the cell during the action potential is rapidly sequestered by the SR and that part of this Ca^{2+} is subsequently released from the SR during the same cardiac cycle.

INTRODUCTION

Based upon ultrastructural considerations (Sommer & Johnson, 1979; Sommer, Bossen & Fabiato, 1982), it has been postulated (Fabiato, 1985*a*, *b*) that Ca^{2+} flux into cardiac cells will not pass directly to the myofilaments but rather will be sequestered by sarcoplasmic reticulum (SR) opposed between the sarcolemma and myofilaments. While some indirect evidence suggests that this may be the case (Reiter, Vierling & Seibel, 1984; Reiter, 1988), a proof of this hypothesis is lacking. The issue is of significance not only in determining the direct impact of Ca^{2+} influx on myofilament activation but also in determining the coupling of Ca^{2+} influx to Ca^{2+}-dependent opening of SR release channels. If a substantial portion of the Ca^{2+} influx via Ca^{2+} channels is pumped into the SR rather than bound to the Ca^{2+} release channel, then the relationship between the magnitude of the L-type calcium current (I_{Ca}) 'trigger' and SR Ca^{2+} release *in situ* would be steeper than previously thought.

We tested the hypothesis that the SR buffers trans-sarcolemmal Ca^{2+} influx during the action potential. As rapid SR Ca^{2+} buffering of Ca^{2+} influx would be masked by a large and rapid SR Ca^{2+} release occurring within milliseconds following activation of I_{Ca}, detection of SR buffering via measurements of Ca^{2+}-dependent fluorescence or contraction requires the abolition of this rapid SR Ca^{2+} release. We devised experiments that deplete the SR of Ca^{2+} and thus prevent the large immediate SR Ca^{2+} release into the cytosolic space following excitation. SR Ca^{2+} depletion prior to electrical stimulation of rested guinea-pig cells was accomplished by two methods. In the first method caffeine is pressure injected from a micropipette onto the cell surface. This depletes the SR Ca^{2+} load, as evidenced by the failure of a subsequent application of caffeine to elicit Ca^{2+} release. Thus, a $[Ca^{2+}]_i$ transient following electrical stimulation of SR Ca^{2+}-depleted cells cannot immediately be derived from the SR. The rate of rise of the $[Ca^{2+}]_i$ transient elicited by an electrical stimulation of such SR Ca^{2+}-depleted cells is slowed and its amplitude is diminished compared to cells in which the SR Ca^{2+} load remains intact. Caffeine applied simultaneously with the onset of depolarization by an action potential in such SR Ca^{2+}-depleted cells,

however, elicits a large, rapid increase in $[Ca^{2+}]_i$. Studies during which Ca^{2+} influx via the L-type Ca^{2+} channels in the SR Ca^{2+}-depleted cells is activated by voltage clamp show that caffeine pulsed onto the cell in conjunction with electrical stimulation does not increase I_{Ca}. That caffeine delivered at the time of an electrical stimulation markedly augments $[Ca^{2+}]_i$ and contraction in cells in which the SR is Ca^{2+} depleted indicates that the SR *rapidly* loads with Ca^{2+} due to Ca^{2+} influx into the cell at the onset of the depolarization, and that this Ca^{2+} that is rapidly pumped into the SR is released by caffeine. That caffeine delivered simultaneously with electrical stimulation in SR Ca^{2+}-depleted cells has this effect indicates that in the absence of caffeine a large fraction of the Ca^{2+} influx during depolarization is rapidly pumped into the SR and is not immediately available to the cytosol or to the myofilaments.

The second model to deplete the SR Ca^{2+} load employs thapsigargin. Thapsigargin is an inhibitor of the SR Ca^{2+} pump in cardiac myocytes (Kirby, Sagara, Gaa, Inesi, Lederer & Rogers, 1992; Wrzosek, Schneider, Grueninger & Chiesi, 1992; Janczewski & Lakatta, 1993). Thus, following sufficient time in thapsigargin, the SR becomes depleted of Ca^{2+}. In addition to causing SR Ca^{2+} depletion thapsigargin prevents SR Ca^{2+} uptake during stimulation. Thus, electrical stimulation of cells during full SR Ca^{2+} pump blockade by thapsigargin elicits a $[Ca^{2+}]_i$ transient and contraction of rested guinea-pig cells that is more rapid and larger in amplitude than those prior to exposure to thapsigargin, permitting the same conclusion as the caffeine depletion model, i.e. that rapid SR sequestration of Ca^{2+} influx occurs during the action potential.

Portions of this work have appeared previously in abstract form (Janczewski, Spurgeon & Lakatta, 1991*a*, *b*).

METHODS

Isolation of myocytes. Single ventricular myocytes were prepared by a modification of the method previously described (Spurgeon *et al.* 1990). Briefly, hearts were quickly removed from male guinea-pigs (300–400 g) anaesthetized with pentobarbitone sodium (50 mg kg^{-1}) and retrogradely perfused for 5 min with a nominally Ca^{2+}-free bicarbonate buffer at 36 ± 1 °C. The perfusion was then switched to a similar solution to which collagenase (1 mg ml^{-1}) and protease (0·04 mg ml^{-1}) had been added. After approximately 10 min of perfusion with this medium, the ventricles were cut off and single cardiac myocytes were mechanically disaggregated. Thereafter, myocytes were rinsed in a bicarbonate solution containing 0·25 mM $CaCl_2$, and finally resuspended in the Hepes buffer containing 1·0 mM Ca^{2+}.

Measurements of indo-1 fluorescence and $Ca^{2+}{}_i$. The experiments were carried out with a modified Zeiss inverted microscope (IM-35) equipped for simultaneous recordings of indo-1 fluorescence, cell length and membrane voltage or current as described elsewhere (Spurgeon *et al.* 1990). The method used simultaneously to measure cell length and changes in intracellular $[Ca^{2+}]_i$ using the fluorescent indicator indo-1 has been described in detail previously (Spurgeon *et al.* 1990; Spurgeon *et al.* 1992). Briefly, myocytes were loaded either with the ester derivative (AM form) of indo-1, or with indo-1 FA (pentapotassium salt) through low-resistance patch electrodes. Both loading of the fluorescent probe and the experiments were done at room temperature (22–24 °C). In myocytes loaded with indo-1 FA, fluorescence transients were calculated by subtracting the background fluorescence emission wavelength, after correction by an algorithm that takes into account the intensity fluctuations of the xenon strobe lamp used for fluorescence excitation (Spurgeon *et al.* 1990). The background fluorescence was measured after establishing the 'giga-seal', but before disruption of the cell membrane within the seal. The cytosolic Ca^{2+} concentration was calculated from an '*in vivo*' calibration curve (Spurgeon *et al.* 1990).

Additional considerations may apply to the interpretation and calibration of indo-1 fluorescence transients in the presence of caffeine. It has been shown recently (O'Neill, Donoso & Eisner, 1990)

that caffeine quenches indo-1 fluorescence equally at both emission wavelengths, while the ratio is maintained. However, cell autofluorescence is not quenched by caffeine (O'Neill *et al.* 1990). Thus, in experiments on indo-1 FA-loaded myocytes, in which the total fluorescence is corrected for autofluorescence, the comparability of the calculated $[Ca^{2+}]_i$ transients is not affected by the presence of caffeine. But, in experiments on indo-1 AM-loaded myocytes, in which the total measured fluorescence is not corrected for autofluorescence, an electrically stimulated $[Ca^{2+}]_i$ transient may produce a slightly different indo-1 fluorescence ratio transient for a given $[Ca^{2+}]_i$ transient in the presence of caffeine, because the relative contribution of the autofluorescence to the total fluorescence at each wavelength increases due to the caffeine-dependent quenching of indo-1. The effect of caffeine on the total fluorescence at each wavelength can be quantified by measuring the caffeine-dependent changes of indo-1 fluorescence emission at each wavelength, provided that the $[Ca^{2+}]_i$ is the same in the presence or absence of caffeine (O'Neill *et al.* 1990). We have made such measurements using cells in which the SR has been depleted of Ca^{2+} (by thapsigargin or by a prior caffeine pulse, cf. Figs 1, 2 and 4) and in which exposure to caffeine does not change $[Ca^{2+}]_i$. The pooled results of the comparison of the fluorescence at the same $[Ca^{2+}]_i$ show that caffeine decreases the fluorescence at each wavelength by roughly 15% ($14{\cdot}1 \pm 1{\cdot}5$% at 490 nm and $13{\cdot}4 \pm 1{\cdot}5$% at 410 nm; mean ± S.E.M., $n = 14$). Thus, the caffeine-dependent quenching of indo-1 may affect the comparability of the indo-1 fluorescence ratio and $[Ca^{2+}]_i$ transients, but only to the extent to which the cell autofluorescence contributes to the total fluorescence. Under our experimental conditions, the cell autofluorescence in myocytes not loaded with indo-1 did not exceed one-tenth of the total fluorescence in myocytes from the same hearts that were loaded with indo-1 AM. Accordingly, the caffeine-dependent quenching of indo-1 should not affect the comparability of the fluorescence ratio transients in indo-1 AM-loaded myocytes by more than about 1·5%.

Length measurements. The bright-field image of the cell was projected onto a 1024 element photodiode array (Model 1024SAQ, Reticon, Salem, MA, USA) as previously described in detail (Spurgeon *et al.* 1990). The array was scanned every 5 ms. The cell edges were detected with user-adjustable trigger circuits, and cell length determined for each scan by digitally counting the diodes between the cell edges.

Whole-cell voltage clamp. Myocytes were voltage clamped using the whole-cell patch clamp method (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). The micropipette electrodes had a resistance of 2–5 MΩ. Membrane currents were recorded using an Axopatch-1A (Axon Instruments, Burlingame, CA, USA) or Axoclamp-2A (Axon Instruments, Foster City, CA, USA) amplifier with the continuous single-electrode voltage clamp circuit.

All experiments were controlled using a VAX 11/730 computer with LPA-11 Lab Interface. The computer was also used to acquire (at 200 Hz), store and analyse the indo-1 ratio, $[Ca^{2+}]_i$, cell length and membrane currents. Rapid, brief (100–200 ms), extracellular applications of caffeine (20 mM in pipette) were injected onto a cell via a micropipette connected to a pressure pulse generator (Picospritzer II, General Valve Corp., Fairfield, NJ, USA) as previously described (Spurgeon *et al.* 1992).

Solutions. The basic superfusing solution contained (mM): NaCl, 137·0; KCl, 5·0; glucose, 15; $MgSO_4$, 1·3; $CaCl_2$, 2·0 and Hepes, 20; pH was adjusted with NaOH to 7·4. In some experiments, the external solution was modified by KCl replacement with 10 mM CsCl. The microelectrode-filling solution contained (mM): KCl, 120; NaCl, 10; Hepes, 20; $MgCl_2$, 1; MgATP, 5. In some experiments, CsCl was used instead of KCl or indo-1 FA (50 μM) was added. pH was adjusted to 7·2 with KOH or, when CsCl was substituted for KCl, with CsOH.

Drugs. Thapsigargin, purchased from Gibco BRL, Grand Island, NY, USA, was dissolved in dimethyl sulphoxide (DMSO) resulting in a final DMSO concentration of < 0·005%. Ryanodine (Conagre, Wind Gap, PA, USA) was added from a 1 mM aqueous stock solution. Caffeine (Sigma Chemical Co., St Louis, MO, USA) was dissolved directly in the standard superfusing solution.

Statistical analysis. Throughout the text average values are given as the means ± S.E.M. A paired Student's *t* test was used to determine the significance of mean differences, assuming significance for $P < 0{\cdot}05$.

RESULTS

Brief caffeine pulses selectively deplete the SR Ca^{2+} load in guinea-pig ventricular myocytes

The objective of the present experiments was to determine whether and to what extent the Ca^{2+} influx *during* an electrical stimulation is pumped directly into the

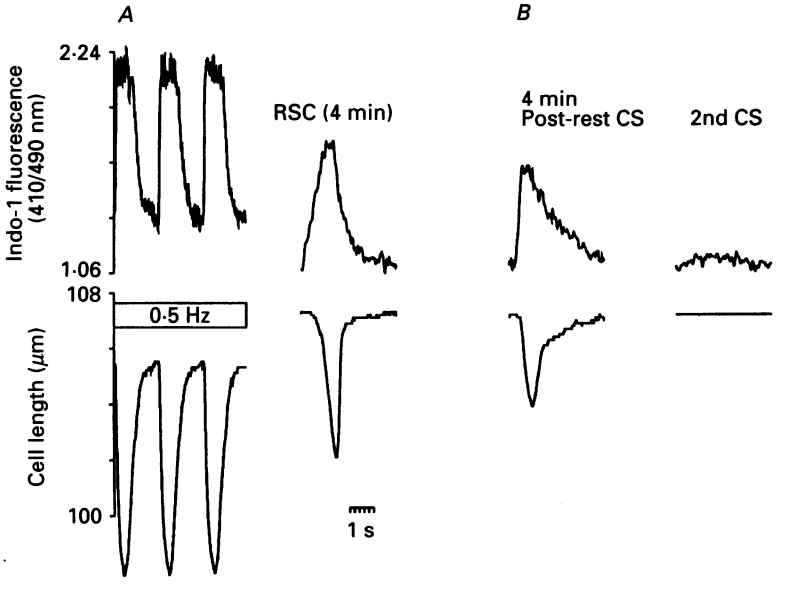

Fig. 1. Effects of a prolonged rest on the electrically stimulated or caffeine-dependent $[Ca^{2+}]_i$ transients, indexed as indo-1 fluorescence ratio (upper traces) and twitch contractions (lower traces) in a representative guinea-pig myocyte loaded with indo-1 AM. *A*, steady-state stimulation (0·5 Hz) is followed by 4 min of rest prior to the generation of an electrically stimulated 'rested-state' contraction (RSC). *B*, after steady-state stimulation and 4 min of rest (as in *A*), a first rapid, brief caffeine pulse (CS), applied from a micropipette placed in the vicinity of the cell, elicits a rapid $[Ca^{2+}]_i$ transient and contraction, and a second CS fails to elicit a $[Ca^{2+}]_i$ transient.

SR. It was essential, therefore, to deplete the SR of Ca^{2+} prior to the electrical stimulation in order to eliminate selectively SR Ca^{2+} release during the time of stimulation-dependent Ca^{2+} entry to the cell. In preliminary experiments we tried to deplete the SR Ca^{2+} load by rest alone. We found, however, that despite a prolonged (4–10 min) rest the SR retained a considerable amount of Ca^{2+}, as evident from the post-rest $[Ca^{2+}]_i$ transients and twitch contractions elicited by caffeine (the first caffeine pulse in Figs 1*B* and 2*A*). Therefore, in an attempt to completely deplete the SR of Ca^{2+}, we pulsed caffeine onto previously rested myocytes. Extracellularly applied caffeine rapidly diffuses into the myocyte (O'Neill *et al.* 1990) and activates the SR Ca^{2+} release channel (Rousseau & Meissner, 1989; Sitsapesan & Williams, 1990). Figure 1*B* (and Fig. 2*A*) shows the effect of repeated caffeine pulses in guinea-pig myocytes, following a 4 min period of rest. The initial exposure of the rested cell to caffeine elicits a $[Ca^{2+}]_i$ transient and contraction. However, a subsequent application of caffeine fails to elicit an increase in $[Ca^{2+}]_i$ or cell shortening. Thus,

under these experimental conditions, the SR is depleted of Ca^{2+} by the initial exposure to caffeine and is not readily replenished in a quiescent myocyte. (In seven cells the average time required for repletion of the SR Ca^{2+} load at rest, as evidenced by the random appearance of feeble $[Ca^{2+}]_i$ transients and contractions in response to a second caffeine pulse, was 5 ± 2 min.) A detectable excitation-independent replenishment of the SR Ca^{2+} content did not occur within at least 180 s in any cell studied.

We also investigated whether the Ca^{2+} pool depleted by caffeine is the same pool released via the SR Ca^{2+} release channel during electrical excitation and whether a caffeine pulse mobilizes Ca^{2+} from cellular Ca^{2+} compartments other than the SR. Pretreatment with 100 nM ryanodine, which latches open the SR Ca^{2+} release channels (Feher & Lipford, 1985; Rousseau, Smith & Meissner, 1987; Bull, Marengo, Suarez-Isla, Sutko & Hidalgo, 1989) and depletes the SR Ca^{2+} load (Hansford & Lakatta, 1986) abolished the caffeine response (not shown). The caffeine-dependent $[Ca^{2+}]_i$ transient and contraction are also abolished during exposure to the SR Ca^{2+} pump inhibitor, thapsigargin (Fig. 4*B*). These results are taken as evidence that a rapid, brief exposure to caffeine, employed in the present experiments, transiently and selectively depletes the SR Ca^{2+} load.

Caffeine-induced SR Ca^{2+} release during the action potential augments electrically stimulated $[Ca^{2+}]$ transients and twitch contractions in cells in which the SR Ca^{2+} load is depleted

The basic experimental protocol employed to determine whether Ca^{2+} influx during depolarization is buffered by the SR is illustrated in Fig. 2*A*. In a rested myocyte, an electrical stimulus (*b* in Fig. 2*A*) applied 30–120 s following a double caffeine pulse (*a* in Fig. 2*A*) elicits a $[Ca^{2+}]_i$ transient and twitch contraction (*b* in Fig. 2*A*) which must originate from the trans-sarcolemmal Ca^{2+} influx during this very beat because the releasable Ca^{2+} SR pool has been depleted prior to the electrical stimulus. The SR was subsequently depleted of Ca^{2+} again, by a caffeine pulse (*c* in Fig. 2*A*). Following another brief period of rest, electrical stimulation was applied simultaneously with the caffeine pulse (*d* in Fig. 2*A*).

Figure 2*B* shows the effect of electrical stimulation in a representative cell in which the SR Ca^{2+} had been depleted prior to excitation. The traces labelled 'CS' were recorded following a double caffeine pulse to ensure that the SR was depleted of Ca^{2+} prior to electrical stimulation. Figure 2*B* shows that the caffeine pulse (CS) fails to elicit an increase in $[Ca^{2+}]_i$ and contraction from the resting level. The traces labelled 'ST' are those in response to a subsequent electrical stimulation. Note that the rise of $[Ca^{2+}]_i$ is slow and the onset of contraction delayed. The difference between traces ST and CS represents an increase in cytosolic Ca^{2+} resulting from Ca^{2+} influx into the cell (less an amount of Ca^{2+} possibly sequestered by the SR and not released during the same cardiac cycle, and Ca^{2+} extrusion via Na^+–Ca^{2+} exchange). Following the electrical stimulation to elicit ST, the cell was double pulsed again with caffeine to deplete any increase in SR Ca^{2+} load that may have resulted from the above manoeuvres (not shown). The traces labelled 'ST+CS' are those measured in response to a combination of an electrical stimulation plus a caffeine pulse delivered simultaneously with excitation. Note the rapid increase and the higher amplitude of the $[Ca^{2+}]_i$ transient and contraction of ST+CS compared to those elicited by

electrical stimulation alone. Since the SR had been depleted of Ca^{2+} prior to the electrical stimulation (as indicated by the lack of response in CS), the difference between ST+CS and ST represents Ca^{2+} taken up by the SR during the action potential following the onset of depolarization. On average, the rate of rise of the

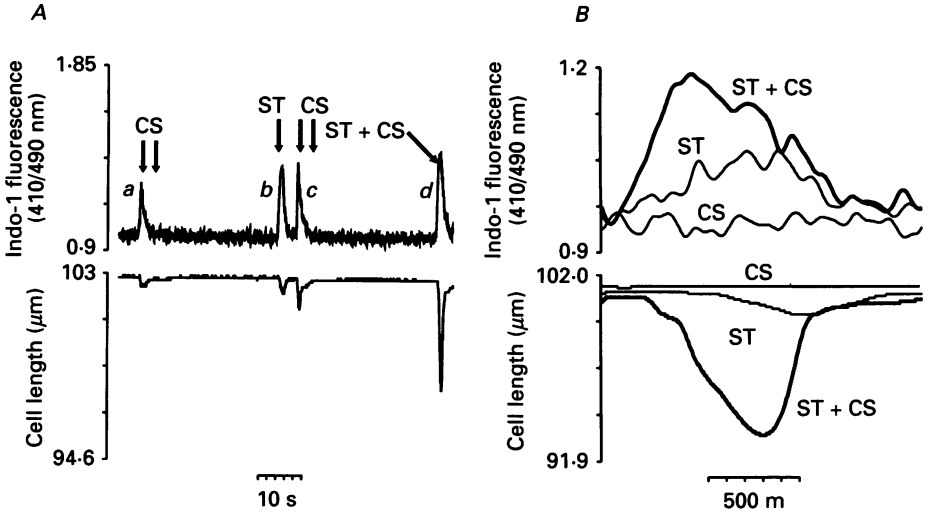

Fig. 2. *A*, a continuous recording of indo-1 fluorescence and cell length illustrates the basic experimental protocol employed in this work. The first of a pair of caffeine 'spritzes' (arrows, CS) onto a rested myocyte depletes the SR of Ca^{2+} (*a* and *c*). Following a double exposure to caffeine and a period of rest, the myocyte is stimulated electrically (ST) or stimulated electrically and simultaneously pulsed with caffeine (ST+CS). *B*, in a different myocyte from that in *A*, superimposed tracings elicited by a caffeine pulse (CS), electrical stimulation (ST) or electrical stimulation plus caffeine (ST+CS). Each of the tracings was obtained after complete SR Ca^{2+} depletion, achieved by brief pulses of caffeine onto the quiescent myocyte.

$[Ca^{2+}]_i$ transient and its amplitude (traces ST+CS *vs.* ST in the type of experiment depicted in Fig. 2) were increased by 53 ± 8 and $63 \pm 9\%$, respectively, while the time integral of the $[Ca^{2+}]_i$ transient was increased by $98 \pm 14\%$ ($n = 21$; $P < 0{\cdot}001$ for each). Recent studies in cardiac myocytes indicate that the SR pumps Ca^{2+} at a rate 3–4 times greater than that expected by the sarcolemmal Na^+–Ca^{2+} exchanger (Bassani, Bassani & Bers, 1992) and that the SR Ca^{2+} uptake is about 2 times more efficient than the combined effects of other Ca^{2+} transport systems involved in the dissipation of the $[Ca^{2+}]_i$ transient (Janczewski & Lakatta, 1993). Thus, in our experiments, the caffeine-dependent augmentation of the rate of rise of the electrically stimulated $[Ca^{2+}]_i$ transient in cells which the SR was depleted of Ca^{2+} prior to excitation primarily reflects the rate of SR accumulation of the trans-sarcolemmal Ca^{2+} influx. Augmentation of the amplitude of the electrically stimulated $[Ca^{2+}]_i$ transient reflects the amount of Ca^{2+} which is accumulated by the SR during the transmembrane Ca^{2+} influx, but not released from the SR into the cytosol during the same cardiac cycle. The significant increases in the rate of rise and the amplitude of $[Ca^{2+}]_i$ transient and contraction by ST+CS *vs.* ST alone indicates that the SR rapidly loads with Ca^{2+} (buffers Ca^{2+} influx) at an early time following the onset of depolarization. A comparison of the ST and ST+CS traces also indicates

that the SR loads *prior to the time* when $[Ca^{2+}]_i$ increases, following the onset of depolarization (ST). The increase in $[Ca^{2+}]_i$ and contraction elicited by electrical stimulation alone (ST) in cells in which the SR is Ca^{2+} depleted prior to excitation may represent a direct access of Ca^{2+} influx into the cytosol. Alternatively, the ST

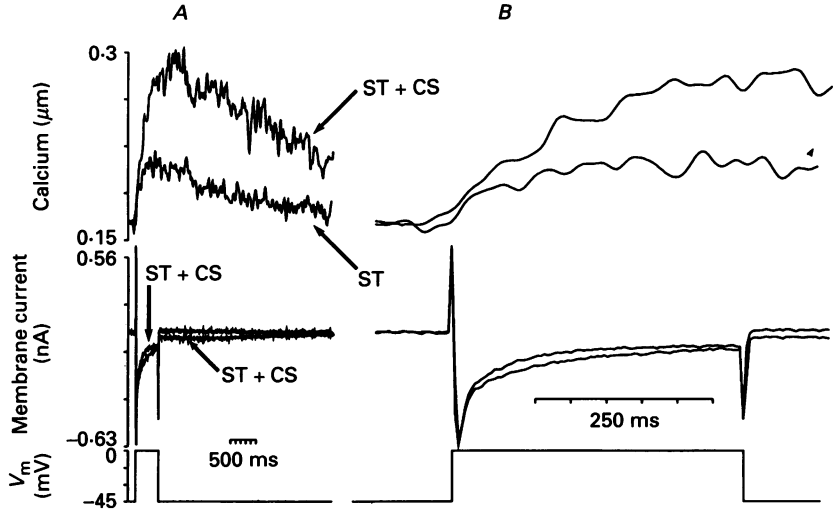

Fig. 3. $[Ca^{2+}]_i$ transient (top), membrane current (middle) and voltage (bottom) in a guinea-pig myocyte loaded with indo-1 FA. *A*, a rapid caffeine pulse applied concomitantly with the depolarizing voltage clamp step (ST + CS) increases the rate of rise and the amplitude of the $[Ca^{2+}]_i$ transient. However, the peak inward current is somewhat diminished while its inactivation is accelerated in the presence of caffeine. The greater magnitude of the inward current *following* repolarization, which corresponds to the augmented $[Ca^{2+}]_i$ transient in the presence of caffeine, probably reflects augmented Ca^{2+} extrusion via $I_{Na,Ca}$ during ST + CS. *B*, the initial part of the same recording (as in *A*), on an expanded time scale.

trace in Fig. 2*B* might not be entirely indicative of a direct access of a portion of Ca^{2+} influx to the cytosol at all, but may reflect Ca^{2+} taken up by the SR immediately following excitation and released later during the same depolarization (see below).

Caffeine pulses do not increase Ca^{2+} entry via I_{Ca}

It might be argued that the combined effects of electrical stimulation and caffeine to augment the $[Ca^{2+}]_i$ transient and contraction in SR Ca^{2+}-depleted cells relative to the effects of electrical stimulation alone do not result entirely from an effect of caffeine to release Ca^{2+} rapidly accumulated by the SR following the onset of stimulation, but in part result from an effect of caffeine to augment Ca^{2+} influx during depolarization, via an augmentation of I_{Ca}. That this is not the case is evident from experiments in which I_{Ca} was measured using the whole-cell voltage clamp method. Figure 3 compares the effect of a caffeine pulse on transmembrane currents and $[Ca^{2+}]_i$ in a cell loaded with indo-1 FA, in which the SR was previously depleted of Ca^{2+} by double caffeine pulses. Note that during the voltage clamp step to 0 mV from a holding potential of −45 mV, the larger and more rapidly rising $[Ca^{2+}]_i$ transient, resulting from the combined effects of depolarization and caffeine, is

accompanied by an inward current of a somewhat smaller peak amplitude and a notably more rapid decay than that in the absence of caffeine. In six additional cells, the peak inward current was either unchanged or decreased, while its inactivation was always accelerated.

Also of note is that following repolarization, a slow decay of the augmented $[Ca^{2+}]_i$ transient in the presence of caffeine is accompanied by an augmented transient inward current, which probably represents increased Ca^{2+} extrusion via the sarcolemmal Na^+–Ca^{2+} exchange (Mechmann & Pott, 1986; Lipp & Pott, 1988). The observation that the augmented transient inward current, attributable to Ca^{2+} extrusion via the Na^+–Ca^{2+} exchange ($I_{Na,Ca}$), accompanies the augmented $[Ca^{2+}]_i$ transient (Fig. 3) indicates that the measured $[Ca^{2+}]_i$ transient, elicited by the combined effects of the excitation-dependent Ca^{2+} influx and the SR Ca^{2+} release by caffeine, probably underestimates the rate of rise and the amplitude of the actual increase in the cytosolic Ca^{2+}, because the higher $[Ca^{2+}]_i$ will favour a high simultaneous Ca^{2+} efflux via the Na^+–Ca^{2+} exchange during the development of the $[Ca^{2+}]_i$ transient. In another five cells we studied the effects of caffeine pulses on I_{Ca} and twitch contractions in myocytes not loaded with the fluorescent Ca^{2+} probe, indo-1 (not shown). These cells were voltage clamped at -40 mV, to eliminate the Na^+ current (I_{Na}), and the outward currents were reduced by Cs^+ replacement of the extracellular and intracellular K^+. These experiments in myocytes depleted of the SR Ca^{2+} with a double caffeine pulse prior to the depolarizing voltage steps, also demonstrate that caffeine expedites the onset, increases the rate of rise and augments the magnitude of the twitch contraction. However, the peak I_{Ca} was decreased and inactivated more rapidly in the presence of caffeine. In these experiments, the decrease in peak I_{Ca} averaged $21 \pm 5\%$. The most important result of the voltage clamp experiments is, however, that neither the peak I_{Ca} nor its time integral is increased by brief caffeine pulses delivered in conjunction with electrical stimulation.

Inhibition of the SR Ca^{2+} uptake by thapsigargin augments the post-rest $[Ca^{2+}]_i$ transient and the twitch contraction

A second strategy to provide information regarding rapid SR sequestration of Ca^{2+} influx during depolarization would be to block the SR Ca^{2+} uptake. If the interpretation of the results of Figs 2 and 3 is correct, i.e. that the SR rapidly buffers Ca^{2+} influx during depolarization, the increase in $[Ca^{2+}]_i$ and contraction elicited by electrical stimulation of a rested myocyte in the presence of the SR pump inhibitor, thapsigargin, ought to be greater than when the SR Ca^{2+} pump is working. Figure 4 illustrates representative examples of experiments in which complete inhibition of the SR pumps by thapsigargin was achieved prior to measurements of the post-rest $[Ca^{2+}]_i$ transients and contractions. In the control (Fig. 4*A*), rapid release of the SR Ca^{2+} load by a caffeine pulse applied in lieu of a steady-state electrical stimulus elicits a brisk $[Ca^{2+}]_i$ transient and contraction. The amplitude of the steady-state $[Ca^{2+}]_i$ transients recovers during the subsequent three to five beats suggesting that three to five depolarizations are necessary to replete the SR Ca^{2+} load. Figure 4*B* shows that following exposure to thapsigargin (200 nM for 20 min), the caffeine pulse fails to trigger the $[Ca^{2+}]_i$ or contractile response, despite prolonged, continuous electrical stimulation between applications of caffeine. (Subsequently measured post-rest

responses to caffeine are also abolished; not shown.) This result indicates that the SR is permanently disabled by thapsigargin, most probably due to an inhibition of SR Ca^{2+} reloading via the SR Ca^{2+} pump (Kirby *et al.* 1992; Wrzosek *et al.* 1992; Janczewski & Lakatta, 1993). In the experiments of the type depicted in Fig. 4, at

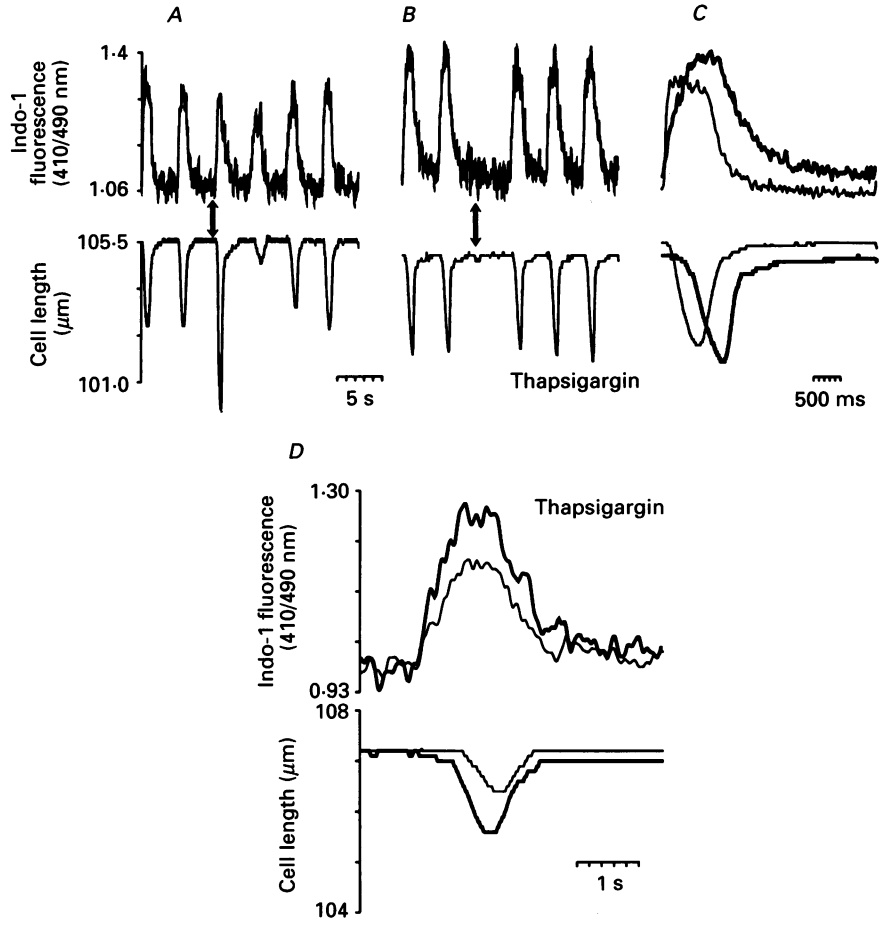

Fig. 4. Effects of thapsigargin on the SR Ca^{2+} load and on electrically stimulated $[Ca^{2+}]_i$ transients and contractions. *A*, in the control, a caffeine pulse (arrow) delivered instead of a steady-state electrical stimulus, triggers a $[Ca^{2+}]_i$ transient and contraction. *B*, this response to caffeine is abolished following 20 min of exposure to 200 nM thapsigargin, indicating SR Ca^{2+} depletion. *C*, averaged beats from *A* and from *B* (bold tracings) are superimposed; note the substantial prolongation of the time to peak of the steady-state $[Ca^{2+}]_i$ transient and contraction after thapsigargin. *D*, effects of thapsigargin on the 'rested-state' beat in this guinea-pig ventricular myocyte. Thin tracings: electrically stimulated $[Ca^{2+}]_i$ transient and contraction following SR Ca^{2+} depletion with caffeine, prior to exposure to thapsigargin. Bold tracings: response to post-rest electrical stimulation after 25 min in thapsigargin. SR inhibition with thapsigargin increases the rate of rise and the amplitude of the 'rested-state' $[Ca^{2+}]_i$ transient and contraction. In contrast to steady-state beats, the time to peak of the post-rest transient is reduced by thapsigargin.

least 15–20 min exposure to 200 nM thapsigargin was required to obtain a complete and permanent removal of the SR function. In some experiments, thapsigargin moderately increased the diastolic $[Ca^{2+}]_i$, presumably due to incomplete relaxation between the markedly prolonged $[Ca^{2+}]_i$ transients. Within 2–3 min of rest after stimulation, however, both the $[Ca^{2+}]_i$ and cell length returned to resting levels similar to these observed prior to exposure to thapsigargin (Fig. 4*A–C vs. D*). Interestingly, complete inhibition of the SR function by thapsigargin did not markedly diminish the steady-state $[Ca^{2+}]_i$ transients and contractions. In fact, quite often the amplitudes of the steady-state beats following thapsigargin were somewhat increased compared to the control (Fig. 4*A–C*). This effect may be attributable, at least in part, to the elevated diastolic $[Ca^{2+}]_i$ during regular stimulation. The most striking effect of the thapsigargin-dependent SR inhibition on steady-state beats was a substantial (more than 100%) prolongation of the times to the peak $[Ca^{2+}]_i$ transient and contraction (Fig. 4*C*).

After establishing that the SR is permanently inhibited by thapsigargin (as in Fig. 4*B*), electrical stimulation was stopped for 2–3 min and the $[Ca^{2+}]_i$ and twitch response to a single post-rest electrical stimulus were measured and compared with the control (pre-thapsigargin) 'rested-state' beat elicited by electrical stimulation following SR Ca^{2+} depletion with caffeine. Figure 4*D* shows that the $[Ca^{2+}]_i$ transient and contraction in response to the electrical stimulation in the presence of thapsigargin are, in fact, larger than in its absence. In seven myocytes, thapsigargin increased the rate of rise and the amplitude of the $[Ca^{2+}]_i$ transient by $65 \pm 21\%$ ($P < 0.01$) and $28 \pm 7\%$ ($P < 0.05$), respectively; the time integral of the $[Ca^{2+}]_i$ transient was increased by $24 \pm 8\%$ ($P < 0.05$). The rate of rise and the amplitude of the accompanying twitch contraction were increased by 57 ± 21 and $112 \pm 27\%$, respectively ($P < 0.01$ for each). It is noteworthy that in contrast to steady-state beats, the times to peak of the post-rest $[Ca^{2+}]_i$ transient and contraction are *decreased* in the presence of thapsigargin.

Following abolition of the SR function by thapsigargin, applications of caffeine during steady-state stimulation (Fig. 4*B*) or at rest (not shown) do not alter $[Ca^{2+}]_i$. Thus, another important finding provided by the experiments using thapsigargin (Fig. 4), and by similar experiments using ryanodine (not shown), is that caffeine releases Ca^{2+} exclusively from the SR, and that the exposures to caffeine employed in the present work do not change the Ca^{2+} buffering capacity of the cell.

Contribution of the SR Ca^{2+} transport to the contraction–relaxation cycle

The results in Figs 2, 3 and 4 strongly suggest that the SR buffers a large fraction of Ca^{2+} influx during the depolarization. Is some of the Ca^{2+} that is pumped into the SR (in an SR Ca^{2+}-depleted cell) during a given depolarization subsequently released into the cytosol during *the same depolarization*? If this were the case, the SR Ca^{2+} load might be expected to become relatively depleted over a portion of the depolarization when the Ca^{2+} influx is waning. Thus, caffeine applied at later times would be expected to release less Ca^{2+} than when applied in conjunction with the electrical stimulus, as in Fig. 2*B*. However, if the SR Ca^{2+} pump is always working to pump back what is released, the 'time window' in which this result would pertain might be expected to be narrow. We examined this by applying caffeine pulses after variable

time intervals from the onset of depolarization, to cells in which the SR was depleted of Ca^{2+} prior to electrical stimulation as in Fig. 2. A representative example of this type of experiment is depicted in Fig. 5. Similar to the results shown above (Figs 2 and 3), caffeine (arrows, bold tracings) applied simultaneously with the trans-

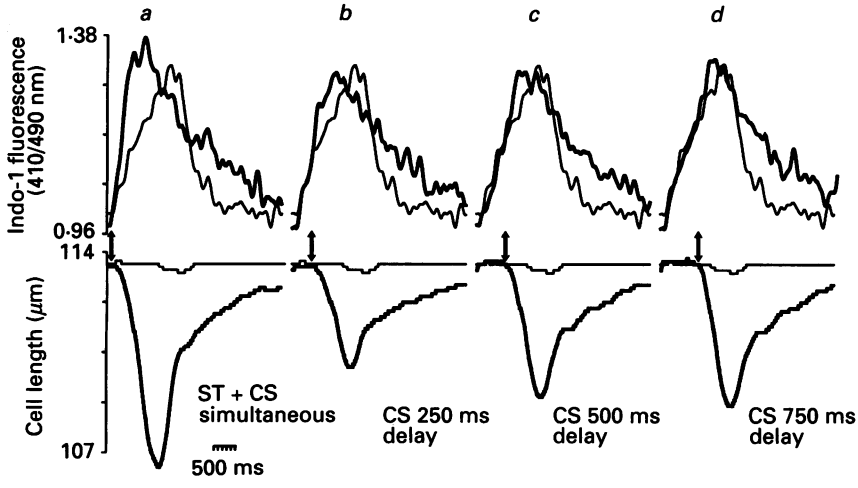

Fig. 5. Effects of caffeine pulses (CS, arrows) applied simultaneously with or after a variable time delay from the onset of the electrical stimulation. Prior to electrical stimulation, the SR of a rested myocyte was depleted of Ca^{2+} by a double caffeine pulse. The control electrically stimulated $[Ca^{2+}]_i$ transient and contraction (thin tracings) are superimposed on recordings (bold tracings) obtained by applying the caffeine pulse (arrows) after the indicated time intervals from electrical stimulation. Electrical stimulation induces the early increase in the $[Ca^{2+}]_i$ transient in both cases. Caffeine applied at variable time intervals shown by arrows, causes the rising phase of the $[Ca^{2+}]_i$ transient to deviate from the control after each application.

sarcolemmal Ca^{2+} influx due to electrical stimulation (tracings *a* in Fig. 5) augments the rate of rise and the magnitude of the $[Ca^{2+}]_i$ transient. However, the caffeine-dependent augmentation of the $[Ca^{2+}]_i$ transients is diminished when caffeine is applied after a delay of 250–750 ms from the electrical stimulation. A small secondary increase in the $[Ca^{2+}]_i$ amplitude also occurs after longer (e.g. 500–750 *vs.* 250 ms) delays from stimulation (tracings *c* and *d* *vs.* *b* in Fig. 5). If the stimulation-dependent increase in cytosolic Ca^{2+} were developing *despite* simultaneous net accumulation of Ca^{2+} by the SR, the augmentation of the $[Ca^{2+}]_i$ transient by caffeine should progressively increase with the time from electrical stimulation. Figure 5 shows that this is not the case, i.e. that the amount of Ca^{2+} releasable from the SR by caffeine (the difference between the rate of rise of ST+CS and ST above) is greatest during, or immediately after the transmembrane Ca^{2+} influx and then decreases with time. Thus, the results depicted in Fig. 5 suggest that during a single cardiac cycle in a cell depleted of SR Ca^{2+} prior to excitation, SR Ca^{2+} sequestration is followed by SR Ca^{2+} release during the same depolarization.

In Fig. 5, (as well as in other experiments presented), the contractile responses to electrical stimulation plus caffeine appear to be potentiated to a greater extent than

the corresponding $[Ca^{2+}]_i$ transients. This effect may be attributable to a higher rate of rise and/or higher amplitude of the $[Ca^{2+}]_i$ transient in the presence of caffeine, because the relationship between $[Ca^{2+}]_i$ and contractile activation is highly non-linear. However, a greater augmentation of the contractile responses may also result,

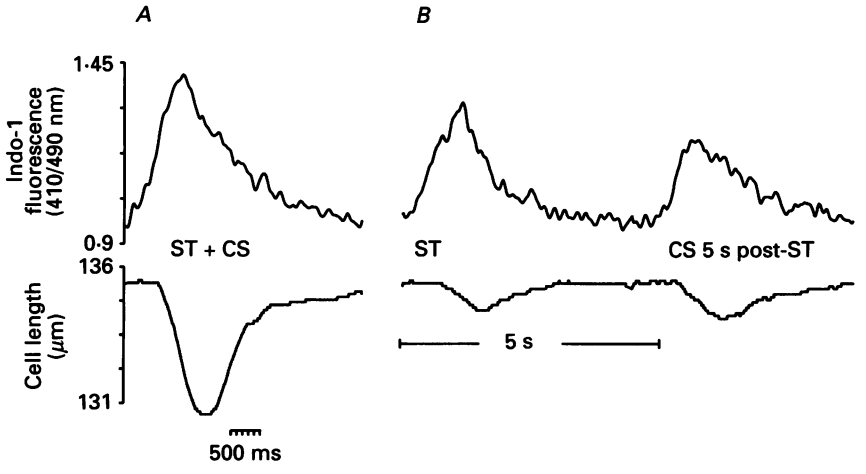

Fig. 6. Estimation of the portion of the net Ca^{2+} influx during the action potential that remains in the SR after completion of the contraction–relaxation cycle in a guinea-pig ventricular myocyte. Stimulation (ST) was preceded by SR Ca^{2+} depletion, achieved by a brief double caffeine pulse, of the rested myocyte (as in Fig. 2). *A*, the magnitude of the $[Ca^{2+}]_i$ response to stimulation plus caffeine (ST + CS) corresponds to the magnitude of the stimulation-dependent net Ca^{2+} influx. *B*, the magnitude of the $[Ca^{2+}]_i$ transient elicited by caffeine pulse (CS) shortly after completion of the electrically stimulated beat (ST), corresponds to the portion of the trans-sarcolemmal Ca^{2+} influx retained in the SR after the cardiac cycle.

at least in part, from the caffeine-dependent increase in myofilament Ca^{2+} sensitivity (Wendt & Stephenson, 1983; Eisner & Valdeolmillos, 1985; de Beer, Grundeman, Wilhelm, Caljouw, Klepper & Schiereck, 1988).

The protocol used in the present study to assess SR Ca^{2+} accumulation during a given excitation–contraction cycle also permits an estimation of how much of the *net* (influx minus simultaneous efflux) Ca^{2+} influx during the cardiac cycle remains in the SR following completion of this cycle, i.e. the amount of Ca^{2+} which is available for a subsequent release (Fig. 6). Under our experimental conditions, the net Ca^{2+} entry into the cytosol during the action potential can be assessed by the amplitude of the $[Ca^{2+}]_i$ transient response to the electrical stimulation when both SR Ca^{2+} uptake and release are prevented. This functional exclusion of the SR is achieved in our experiments by caffeine depletion of SR Ca^{2+} prior to depolarization, and application of caffeine simultaneously with a subsequent depolarization (Fig. 6*A*). The SR Ca^{2+} content following completion of the normal cardiac cycle (lefthand side of the tracings in Fig. 6*B*) can be indexed as the amplitude of the $[Ca^{2+}]_i$ transient elicited by caffeine following the completion of the cardiac cycle (part of the tracings on the far right in Fig. 6*B*). The results using this method indicate that in guinea-pig ventricular myocytes stimulated after the SR Ca^{2+} depletion, $67 \pm 5\%$ ($n = 9$) of the

net Ca^{2+} influx during the action potential remains in the SR following completion of the contraction–relaxation cycle.

DISCUSSION

Our results show that in cells in which the SR is depleted of Ca^{2+} prior to excitation, and in which the SR accumulation of Ca^{2+} upon depolarization is abolished by a caffeine pulse administered simultaneously with depolarization or by bathing cells in thapsigargin, the rates of rise and the amplitudes of the electrically stimulated, post-rest $[Ca^{2+}]_i$ transient and twitch contraction are substantially increased. These results suggest that the SR rapidly buffers the trans-sarcolemmal Ca^{2+} influx elicited by depolarization. However, this conclusion can be confirmed only after showing that the experimental manoeuvres designed to deplete the SR Ca^{2+} load prior to depolarization and to inhibit SR Ca^{2+} accumulation during depolarization do not appreciably affect other Ca^{2+} transport systems in a manner which could mimic the SR-dependent effects.

Caffeine pulses selectively disable the SR

For the present experiments it was essential to create an experimental model in which the SR is completely depleted of Ca^{2+} prior to electrical stimulation, so that the stimulation-dependent changes in the cytosolic Ca^{2+} would result solely from the trans-sarcolemmal Ca^{2+} influx, minus the amount of Ca^{2+} possibly pumped to the SR and not released during the same cardiac cycle. It was also essential that functional removal of the SR during stimulation be selective, so that other cellular Ca^{2+} transport systems could not notably contribute to $[Ca^{2+}]_i$ changes (specifically to increase $[Ca^{2+}]_i$) during electrical stimulation plus caffeine.

We achieved complete, stable SR Ca^{2+} depletion by pulsing caffeine onto previously rested myocytes (Figs 1 and 2). That caffeine selectively mobilizes Ca^{2+} from the SR was confirmed by lack of responses to caffeine pulses following functional removal of the SR by the action of specific SR inhibitors ryanodine (not shown) or thapsigargin (Fig. 4*B*). The thapsigargin-dependent abolition of the $[Ca^{2+}]_i$ and contractile responses to caffeine pulses applied instead of steady-state electrical stimuli also demonstrates that brief exposures to caffeine employed in the present work do not decrease the Ca^{2+} binding capacity of the cell. Thus, augmentation of the electrically stimulated $[Ca^{2+}]_i$ transient by the caffeine pulse results exclusively from its effect of rapidly releasing Ca^{2+} from the SR.

Another issue to be considered, at least theoretically, is that caffeine applied in conjunction with an electrical stimulation could promote additional Ca^{2+} release from an SR compartment that had not been completely depleted of Ca^{2+} by caffeine given prior to the electrical stimulation. If electrical stimulation in the presence of caffeine were to mobilize additional Ca^{2+} from an SR compartment not depleted by caffeine given prior to the stimulation, a *subsequent* electrically stimulated $[Ca^{2+}]_i$ transient in the presence of caffeine ought to be less than that elicited following a prior caffeine exposure in the absence of electrical stimulation. However, our experiments show that caffeine prior to stimulation depletes the SR of Ca^{2+} to the same extent as when it is applied in conjunction with electrical stimulation; subsequent responses to caffeine administered in the absence of stimulation are

abolished while the $[Ca^{2+}]_i$ transients elicited by electrical stimulation plus caffeine are virtually the same. Additionally, a hypothetical SR Ca^{2+} compartment not depleted by an initial caffeine application would be expected to discharge Ca^{2+} in response to caffeine applied at later times during the development of the electrically stimulated $[Ca^{2+}_i]$ transient. The experiments of the type depicted in Fig. 5 show that the electrically stimulated Ca^{2+} transients are markedly augmented by caffeine only when caffeine is applied simultaneously or shortly after the electrical stimulation (tracings *a* in Fig. 5), but not following longer ($\geqslant 250$ ms) intervals between stimulation and the caffeine pulse. Thus, it is reasonable to assume that under our experimental conditions, caffeine applied in conjunction with electrical stimulation releases Ca^{2+} from the same SR compartment that is completely depleted of Ca^{2+} by caffeine applied prior to electrical stimulation.

Brief, rapid exposures to caffeine do not increase the trans-sarcolemmal Ca^{2+} influx

During the action potential or the depolarizing voltage clamp step to 0 mV, as in the present experiments, Ca^{2+} enters the myocyte via I_{Ca} and, possibly, via the Na^+–Ca^{2+} exchange (Barcenas-Ruiz, Beuckelmann & Wier, 1987; Mitchell, Powell, Terrar & Twist, 1987; Beuckelmann & Wier, 1988; Doerr, Denger, Doerr & Trautwein, 1990; Leblanc & Hume, 1990). It has been reported that caffeine increases the cellular cyclic AMP level due to an inhibition of cAMP phosphodiesterase (Butcher & Sutherland, 1962; Sholtz, 1984). This in turn may increase Ca^{2+} entry via I_{Ca}, because of the cyclic AMP-dependent phosphorylation of the Ca^{2+} channel proteins (Kemeyama, Hescheler, Hofmann & Trautwein, 1986). Therefore, using whole-cell voltage clamp, we tested whether and in what manner the brief exposures to caffeine might modify Ca^{2+} influx via the L-type Ca^{2+} channels. The consistent observation in that type of experiment was that brief caffeine pulses as employed in our experiments do not increase I_{Ca}. In fact, the peak I_{Ca} was often reduced. The extent of this reduction presumably depended on the timing between the commencement of the cellular effects of caffeine and the onset of I_{Ca}. Calcium influx via I_{Ca} consistently decayed faster in the presence of caffeine (Fig. 3). Diminution of Ca^{2+} influx via I_{Ca} during the caffeine pulse may reflect the $[Ca^{2+}]_i$-dependent inactivation of I_{Ca} (Eckert & Chad, 1984; Lee, Marban & Tsien, 1985; Chad & Eckert, 1986; Boyett, Kirby & Orchard, 1988) and/or a direct blocking effect of caffeine on Ca^{2+} channels (Hughes, Hering & Bolton, 1990; Zholos, Baidan & Shuba, 1991). Our observations of the action of caffeine pulses on I_{Ca} are in agreement with recent reports on guinea-pig ventricular myocytes in which the peak I_{Ca} was unchanged while the inactivation by a relatively brief (2–8 s) exposure to 5 mM caffeine was accelerated (Doerr *et al.* 1990). Similarly, the amplitude of I_{Ca} was decreased in guinea-pig atrial cells pulsed with 1 mM caffeine (Lipp, Pott, Callewaert & Carmeliet, 1992). Caffeine has also been reported to decrease Ca^{2+} current in cardiac Purkinje fibres (Eisner, Lederer & Noble, 1979; Hess & Wier, 1984). On the other hand, the reported increase in the peak I_{Ca} during prolonged caffeine exposure that has previously been interpreted to occur via change in cAMP (Sipido & Wier, 1991) may reflect a longer time required for the cyclic AMP-dependent kinase to phosphorylate the sarcolemmal Ca^{2+} channel.

There is no indication in the literature that caffeine directly affects the sarcolemmal Na^+–Ca^{2+} exchanger. The magnitude and the direction of the sarcolemmal transport

of Ca^{2+} in exchange for Na^+ is, however, dependent on the transmembrane potential and gradients for Na^+ and Ca^{2+} (Mullins, 1979; Mechmann & Pott, 1986). Under the present experimental conditions, the extracellular concentrations of Ca^{2+} and Na^+ (in the superfusing solution) are considered to be constant. Thus, the transmembrane gradients for these ions will depend on intracellular concentrations, which may be altered during a single post-rest beat by the action of caffeine on the stimulation-dependent influx of Ca^{2+} and/or Na^+, and on the SR Ca^{2+} release. Caffeine pulses employed in the present work diminish rather than augment Ca^{2+} influx via the L-type channel (see above). Also, in the presence of caffeine, sodium influx during the action potential, and thus the intracellular Na^+ concentration during the development of the $[Ca^{2+}]_i$ transient, may be expected to be somewhat reduced because of a caffeine-dependent inhibition of I_{Na} (Hambuchi, Tanaka, Furukawa & Tsujimura, 1991). This would probably decrease rather than increase the amplitude of the $[Ca^{2+}]_i$ transient (Leblanc & Hume, 1990). In our experiments, caffeine augments the $[Ca^{2+}]_i$ transients elicited by action potentials (Figs 2, 5 and 6), as well as those stimulated under whole-cell voltage control (Fig. 3). Thus, the effects of caffeine on the Na^+–Ca^{2+} exchange, which might originate from caffeine-dependent alterations of the membrane potential during an action potential, do not qualitatively affect our results.

Caffeine may, however, alter the electrogenic Na^+–Ca^{2+} exchange by altering the configuration of the action potential, and therefore affect, quantitatively, the comparability of the action potential-dependent responses. Doerr *et al.* (1990) reported that a relatively brief (2 s) application of caffeine moderately lengthens the action potential duration, while 8 s exposures decrease the plateau height and prolong the final repolarization. They attributed the effects of caffeine on the plateau level to an outward current via the Ca^{2+}-activated non-specific cation channel (Colquhoun, Neher, Reuter & Stevens, 1981) while the prolonged action potential duration was attributed to an increased Ca^{2+} efflux via $I_{Na,Ca}$. We have observed that rapid, brief (100–200 ms) pulses of caffeine in guinea-pig cells either do not affect or accelerate the plateau repolarization of the action potential, but prolong the final repolarization phase, concomitant with the decay of the augmented $[Ca^{2+}]_i$ transients (A. M. Janczewski & E. G. Lakatta, unpublished observations). We interpret the diminution of the plateau as the result of the caffeine-dependent diminution of I_{Ca} (see above) and, possibly, due to activation of the non-specific cation channel, as suggested by Doerr *et al.* (1990). Prolongation of the final repolarization is consistent with an inward current generated by an electrogenic Na^+–Ca^{2+} exchange during the augmented $[Ca^{2+}]_i$ transient (Fig. 3; Barcenas-Ruiz *et al.* 1987; Doerr *et al.* 1990). This interpretation is supported by the observation that the action potential is abbreviated, due to the accelerated repolarization in the plateau range, when a brief caffeine pulse is applied onto guinea-pig ventricular myocytes in which the $[Ca^{2+}]_i$ transients are abolished by dialysis with 20 mM EGTA (A. M. Janczewski & E. G. Lakatta, unpublished observations). Therefore, we conclude that under the present conditions, caffeine should not affect the action potential configuration to increase Ca^{2+} influx (or decrease Ca^{2+} efflux) via the Na^+–Ca^{2+} exchange. Rather, the caffeine-dependent augmentation of the $[Ca^{2+}]_i$ transient modulates the action potential configuration.

Another issue concerning Na^+–Ca^{2+} exchange is that the $[Ca^{2+}]_i$ increase, measured as the $[Ca^{2+}]_i$ transient elicited by the combined effects of electrical stimulation and simultaneous application of caffeine, may underestimate the actual magnitude and rate of rise of the $[Ca^{2+}]_i$ transient, because the higher $[Ca^{2+}]_i$ will favour simultaneous Ca^{2+} efflux via the Na^+–Ca^{2+} exchange. This conclusion is supported by the observation that the augmented transient inward current, attributable to Ca^{2+} efflux via $I_{Na,Ca}$, accompanies the augmented $[Ca^{2+}]_i$ transients (Fig. 3). In summary, caffeine should not increase Ca^{2+} entry via $I_{Na,Ca}$, but in contrast Na^+–Ca^{2+} exchange is likely to contribute to a diminution of the $[Ca^{2+}]_i$ responses in the presence of caffeine. Thus, the difference between the ST and ST + CS tracings in Figs 2 and 3, which indicates rapid SR pumping of Ca^{2+} influx, actually underestimates the amount of Ca^{2+} buffered by the SR.

Selective inhibition of the SR Ca^{2+} uptake augments the post-rest $[Ca^{2+}]_i$ transients and twitch contractions

Thapsigargin, a sesquiterpene lactone, (Rassmussen, Christensen & Sandberg, 1978; Thastrup, 1990), has been shown to block the SR function in cardiac myocytes by selectively inhibiting the SR Ca^{2+} uptake (Kirby *et al.* 1992; Wrzosek *et al.* 1992; Janczewski & Lakatta, 1993). The effect of thapsigargin is specific to the Ca^{2+}-dependent ATPase of the SR, i.e. it affects neither the Ca^{2+}-dependent ATPase nor the Na^+–K^+-ATPase of the plasma membrane. (Lytton, Westlin & Hanley, 1991; Sagara & Inesi, 1991; Kirby *et al.* 1992; Wrzosek *et al.* 1992). The present experiments show that in guinea-pig ventricular myocytes, at least 15 min of exposure to 200 nM thapsigargin is necessary to completely and permanently remove the SR function (Fig. 4*A* and *B*). Similar or longer times of exposure to submicromolar concentrations of thapsigargin were required to inhibit the SR in intact rat ventricular myocytes (Kirby *et al.* 1992; Wrzosek *et al.* 1992; Janczewski & Lakatta, 1993). These observations contrast with a much more rapid action of thapsigargin to inhibit the SR Ca^{2+} pump in broken cell cardiac preparations (e.g. Kirby *et al.* 1992; Wrzosek *et al.* 1992). Under the present experimental conditions thapsigargin significantly increased the rates of rise and the amplitudes of the post-rest $[Ca^{2+}]_i$ transients and twitch contractions (Fig. 4*D*). This effect results from an inhibition of the SR Ca^{2+} uptake rather than from a modified trans-sarcolemmal Ca^{2+} influx or the SR Ca^{2+} release, because thapsigargin does not directly affect I_{Ca} in rat ventricular myocytes (Kirby *et al.* 1992), and because under our experimental conditions the SR was permanently deprived of Ca^{2+} after about 15–20 min in 200 nM thapsigargin (Fig. 4*B*). It is likely, however, that when the SR Ca^{2+} uptake is inhibited by thapsigargin, the transmembrane Ca^{2+} influx during depolarization is diminished, due to the $[Ca^{2+}]_i$-dependent inactivation of I_{Ca} and due to a smaller transmembrane gradient for Ca^{2+} influx via $I_{Na,Ca}$ as noted above.

Changes in the SR Ca^{2+} content during the contraction–relaxation cycle

The experimental protocol in which the SR is deprived of Ca^{2+} prior to stimulation, and in which the SR Ca^{2+} load can be rapidly discharged (with caffeine) after a variable delay from the onset of electrical stimulation, permits assessment of changes in the SR Ca^{2+} content during the entire contraction–relaxation cycle. Our results

show that in SR Ca^{2+}-depleted cells, at least 50% of Ca^{2+} influx during the action potential is rapidly sequestered by the SR (e.g. Figs 2*B* and 4*D*). The remaining portion of Ca^{2+} influx may directly access the cytosol, or may be pumped into the SR and re-released, so that this portion of SR Ca^{2+} accumulation is not detectable using caffeine. We interpret our results to indicate that Ca^{2+} which is accumulated by the SR during the early depolarization period is, in part at least, subsequently released from the SR during the same depolarization. This is evidenced by the observations (Fig 5) that the SR Ca^{2+} load is highest during or immediately after the transmembrane Ca^{2+} entry and decreases during the development of the electrically stimulated $[Ca^{2+}]_i$ transient. These observations support the suggestion of Reiter *et al.* (1984) that Ca^{2+} influx is rapidly sequestered by the SR and subsequently released from the SR during the same action potential.

Based upon studies on isolated guinea-pig ventricular preparations (for review see Reiter, 1988) it has been suggested that the delayed onset of the 'rested-state' contraction indicates a delay necessary for Ca^{2+} transport from the putative 'uptake sites' to the 'release sites' of the SR. Our results show that single myocytes of guinea-pig heart, depleted of the SR Ca^{2+} by a prolonged rest (Fig. 1*A*) or by rest and caffeine (e.g. Fig. 2*B*) also typically demonstrate delayed twitch development. However, simultaneous measurements of the $[Ca^{2+}]_i$ transient and cell shortening (Figs 1*A*, 2*B*, 4*D*, 5 and 6*B*) clearly show that the $[Ca^{2+}]_i$ transient commences immediately following the onset of stimulation. This indicates that the peculiar configuration of the 'rested-state' contraction in guinea-pig ventricular myocardium results from a non-linearity between $[Ca^{2+}]_i$ and contractile activation, rather than from purported Ca^{2+} shifts within the SR.

Our experiments also permit an assessment of how much of the net Ca^{2+} influx during the action potential is retained by the SR following completion of the cardiac cycle. A relative estimate of the net Ca^{2+} influx was obtained from the amplitude of the electrically stimulated $[Ca^{2+}]_i$ transient in the absence of a functional SR (Fig. 6*A*). Since the augmented $[Ca^{2+}]_i$ transients resulting from the SR inhibition probably induce greater Ca^{2+} extrusion via the Na^+–Ca^{2+} exchanger (see above), this method of assessing Ca^{2+} influx underestimates the amount of Ca^{2+} entering the myocyte during a normal beat (Fig. 6*B*), i.e. when the SR is not disabled. Using the amplitude of the caffeine-dependent $[Ca^{2+}]_i$ transient elicited briefly after completion of the contraction–relaxation cycle (Fig. 6*B*) as an index of Ca^{2+} remaining in the SR following the cycle, we estimated that about 60–70% of the net Ca^{2+} influx in SR Ca^{2+}-depleted guinea-pig myocytes is retained in the SR. The closest estimates of that type in guinea-pig ventricular myocytes were reported by Bers, Bridge & Spitzer (1989), who assessed the SR Ca^{2+} reaccumulation from the magnitude of consecutive $[Ca^{2+}]_i$ transients elicited by rapid cooling. They found that about 50–80% of the released Ca^{2+} is reaccumulated by the SR, while the remaining portion of Ca^{2+} is extruded from the cell via the Na^+–Ca^{2+} exchanger. Similarly, Crespo, Grantham & Cannell (1990) reported that the SR Ca^{2+} sequestration and the Na^+–Ca^{2+} exchange accounts for about 85 and 15%, respectively, of the $[Ca^{2+}]_i$ transient relaxation in guinea-pig ventricular myocytes.

Conclusions

After examination of the factors that could affect the interpretation of our results, we draw the following conclusions. First, in guinea-pig ventricular myocytes a substantial portion of the trans-sarcolemmal Ca^{2+} influx during post-rest stimulation is rapidly pumped to the SR rather than directly accessing the cytosol and activating the myofilaments (Figs 2–5). Our results support the idea (Fabiato, 1985*a*, *b*) that upon depolarization Ca^{2+} flux into cardiac cells will not pass directly to the myofilaments but rather will be buffered by the SR apposed between the sarcolemma and myofilaments (Sommer & Johnson, 1979; Sommer *et al.* 1982). Second, a part of the calcium influx accumulated within the SR is subsequently released to the cytosol during the same depolarization (Fig. 5). This result is consistent with the previous hypothesis of Reiter *et al.* (1984). Third, approximately 60–70% of Ca^{2+} flux into guinea-pig myocytes depleted of the SR Ca^{2+} prior to a depolarization is retained in the SR following completion of the cardiac cycle (Fig. 6).

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