Ca^{2+} Removal Mechanisms in Rat Cerebral Resistance Size Arteries

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ABSTRACT Tissue blood flow and blood pressure are each regulated by the contractile behavior of resistance artery smooth muscle. Vascular diseases such as hypertension have also been attributed to changes in vascular smooth muscle function as a consequence of altered Ca^{2+} removal. In the present study of Ca^{2+} removal mechanisms, in dissociated single cells from resistance arteries using fura-2 microfluorimetry and voltage clamp, Ca^{2+} uptake by the sarcoplasmic reticulum and extrusion by the Ca^{2+} pump in the cell membrane were demonstrably important in regulating Ca^{2+}. In contrast, the Na^+-Ca^{2+} exchanger played no detectable role in clearing Ca^{2+}. Thus a voltage pulse to 0 mV, from a holding potential of −70 mV, triggered a Ca^{2+} influx and increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). On repolarization, $[Ca^{2+}]_i$ returned to the resting level. The decline in $[Ca^{2+}]_i$ consisted of three phases. Ca^{2+} removal was fast immediately after repolarization (first phase), then plateaued (second phase), and finally accelerated just before $[Ca^{2+}]_i$ returned to resting levels (third phase). Thapsigargin or ryanodine, which each inhibit Ca^{2+} uptake into stores, did not affect the first but significantly inhibited the third phase. On the other hand, Na^+ replacement with choline$^+$ did not affect either the phasic features of Ca^{2+} removal or the absolute rate of its decline. Ca^{2+} removal was voltage-independent; holding the membrane potential at 120 mV, rather than at −70 mV, after the voltage pulse to 0 mV, did not attenuate Ca^{2+} removal rate. These results suggest that Ca^{2+} pumps in the sarcoplasmic reticulum and the plasma membrane, but not the Na^+-Ca^{2+} exchanger, are important in Ca^{2+} removal in cerebral resistance artery cells.

INTRODUCTION

Because the principal trigger for contraction of smooth muscle is an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), mechanisms generating increases in $[Ca^{2+}]_i$ have been subject to intensive investigation. Depending on the stimulus, a rise in $[Ca^{2+}]_i$ may reflect Ca^{2+} entry from the outside, release from an internal store, or both. Ca^{2+} entry across the plasma membrane occurs through Ca^{2+} channels. In smooth muscle, three types of Ca^{2+} channel exist: a low-voltage, rapidly inactivating, small conductance channel (T-type), and a dihydropryidine-sensitive, high-threshold, large conductance channel (L-type; Benham et al., 1987; Vivaudou et al., 1988). A third type of Ca^{2+} channel directly operated by agonists and insensitive to voltage has also been proposed (Benham and Tsien, 1987). In addition to Ca^{2+} fluxes across the plasmalemma, Ca^{2+} may also be released from an internal store by agonist-generated IP_3 (Horowitz et al., 1996) or by a Ca^{2+}-induced Ca^{2+} release mechanism (Kamishima and McCarron, 1997).

After stimulation $[Ca^{2+}]_i$ returns to the resting levels, permitting relaxation. However, although their importance is well appreciated, the exact efflux or uptake routes of Ca^{2+} removal in smooth muscle remain unclear. Four transport systems are thought to be involved in Ca^{2+} removal. A Ca^{2+} pump and a Na^+-Ca^{2+} exchanger in the plasma membrane are believed to extrude Ca^{2+}, whereas a Ca^{2+} pump in sarcoplasmic reticulum (SR) and a Ca^{2+} uniporter in mitochondria sequester Ca^{2+}. In cardiac myocytes, Ca^{2+} removal occurs largely by Ca^{2+} pumps in the SR and, to a lesser extent, by a Na^+-Ca^{2+} exchanger in the sarcolemma (e.g., Wier, 1990); the contributions of Ca^{2+} pumps in the cell membrane and the Ca^{2+} uniporter in the mitochondria are relatively small (Bassani et al., 1992). In smooth muscle, however, the Ca^{2+} removal pathways are less well defined. This prompted the present study, in which Ca^{2+} removal mechanisms have been examined in resistance artery smooth muscle. Evidence is provided that Ca^{2+} pumps in the SR and cell membrane, but not the Na^+-Ca^{2+} exchanger, are important in Ca^{2+} removal. Part of the study has already been published as an abstract (McCarron and Kamishima, 1997).

MATERIALS AND METHODS

Drugs and statistics

Fura-2 pentapotassium salt was purchased from Molecular Probes (Eugene, OR), and Bay K 8644, ryanodine, and thapsigargin were from Calbiochem-Novabiochem (Nottingham, England). Bay K 8644 was dissolved in ethanol to make a 1 mM stock solution. The final concentration of ethanol in bathing solution was 0.05%. Ryanodine and thapsigargin were dissolved in dimethyl sulfoxide (DMSO) to produce stock solutions of 30 mM and 500 μM, respectively. The final concentration of DMSO in experimental solutions was 0.1% in both cases. Dithioerythritol, collagenase Type F, and hyaluronidase Type I-S were purchased from Sigma Chemical (Dorset, England). Papain was obtained from Worthington Biochemical Corporation (Freehold, NJ). When appropriate, the data were expressed as means ± SEM of n cells, and significant difference was detected using Student's unpaired or paired t-test ($p < 0.05$).

Received for publication 30 December 1997 and in final form 1 June 1998.

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0006-3495/98/10/1767/07 $2.00

Cell dissociation

Male Sprague-Dawley rats (225–350 g) were killed by pentobarbitone sodium overdose (150 mg kg^{-1}, I.P.). The brain was removed and placed in a solution containing (mM) 137 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 11 glucose (pH adjusted to 7.4 with NaOH). Superior cerebral arteries (diameter $\approx$ 150 μm) were dissected, and single smooth muscle cells were dissociated as previously described (Quayle et al., 1994). Briefly, a low-Ca^{2+} solution was used for cell dissociation (mM): 80 Na glutamate, 54 NaCl, 5 KCl, 1 $MgCl_2$, 0.1 $CaCl_2$, 10 HEPES, 10 glucose, and 0.2 EDTA. pH of the low-Ca^{2+} solution was adjusted to 7.3 at room temperature with NaOH to provide pH 7.4 at 35°C. The arteries were first treated with (mg ml^{-1}) 1.7 papain and 0.7 dithioerythritol for 30 min at 35°C, then further digested with (mg ml^{-1}) 1.7 type F collagenase and 1 type I-S hyaluronidase for 20 min. The arteries were then rinsed with enzyme-free low-Ca^{2+} solution, and single cells were dispersed by triturating the arteries with a fire-polished Pasteur pipette. The cell suspension was stored in a refrigerator and used the same day.

Voltage-clamp technique

Command pulses were applied in tight-seal whole-cell recording mode (Hamill et al., 1981). Unless otherwise stated, the extracellular solution consisted of (mM) 80 Na glutamate, 40 NaCl, 20 tetraethylammonium (TEA) chloride, 1.1 $MgCl_2$, 3 $CaCl_2$, 10 HEPES, and 10 glucose (pH adjusted to 7.4 with NaOH). In most cases, the composition of the pipette solution was (in mM) 145 CsCl, 3 $MgCl_2$, 3 Na_2ATP, 10 HEPES, and 0.04 Fura-2 pentapotassium salt (pH adjusted to 7.2 with CsOH). Where the role of Na^+-Ca^{2+} exchanger was studied, Na^+ was eliminated from both the bathing and the pipette solutions. Hence, the composition of the bathing solution was (mM) 120 choline Cl, 20 TEA Cl, 1.1 $MgCl_2$, 3 $CaCl_2$, 10 HEPES, and 10 glucose (pH adjusted to 7.4 with CsOH). The composition of the pipette solution was (mM) 145 CsCl, 3 MgATP, 10 HEPES, and 0.04 Fura-2 (pH adjusted to 7.2 with CsOH). Ryanodine was added to the pipette solution, and thapsigargin was applied to both pipette and bathing solutions. Whole-cell currents were amplified with an Axopatch 1D (Axon Instruments, Foster City, CA), filtered at 500 Hz, and sampled at 1.5 kHz with pCLAMP software (version 6.0.1; Axon Instruments). In the standard protocol, a 1.6-s voltage pulse to 0 mV was applied from a holding potential of -70 mV to trigger Ca^{2+} influx (I_{Ca}) through voltage-dependent Ca^{2+} channels. All experiments were performed at room temperature (18°C-22°C).

Ca^{2+} microfluorimetry

High temporal $[Ca^{2+}]_i$ measurement was carried out using a PTI deltascan (Photon Technology International, London) as described previously (Kamishima and McCarron, 1996). Cells were dialyzed with a pipette solution containing 40 μM membrane-impermeable Fura-2 pentapotassium salt. Cells were illuminated with alternating UV light (340/380 nm; 9-nm bandpass) at 100 Hz, and emission signals were obtained at 510 nm (60-nm bandpass, complete ratio obtained at 50 Hz). The K_d for Fura-2 was determined as 280 nM from an in vitro calibration (Kamishima and McCarron, 1996), as were R_{min} and R_{max}; the latter were decreased by 15% to make up for the viscosity of intracellular milieu (Poenie, 1990). Background fluorescence was measured when the tight seal was formed, but before achieving whole-cell configuration and subtracted from the fluorescent counts during the experiments.

Calculation of Ca^{2+} removal rate

Unless otherwise stated, a depolarizing pulse to 0 mV was given from a holding potential of -70 mV. Upon repolarization to -70 mV, the increased $[Ca^{2+}]_i$ returned to the basal level (Fig. 1). The declining phase of the Ca^{2+} transient was fitted with high-order polynomial equations, and the Ca^{2+} removal rate was calculated as the negative derivative of the polynomial by averaging the slope of two adjacent data points (Kamishima and McCarron, 1996). The calculated Ca^{2+} removal rate was expressed either as a function of time, where time = 0 is the instant of repolarization, or of $[Ca^{2+}]_i$.

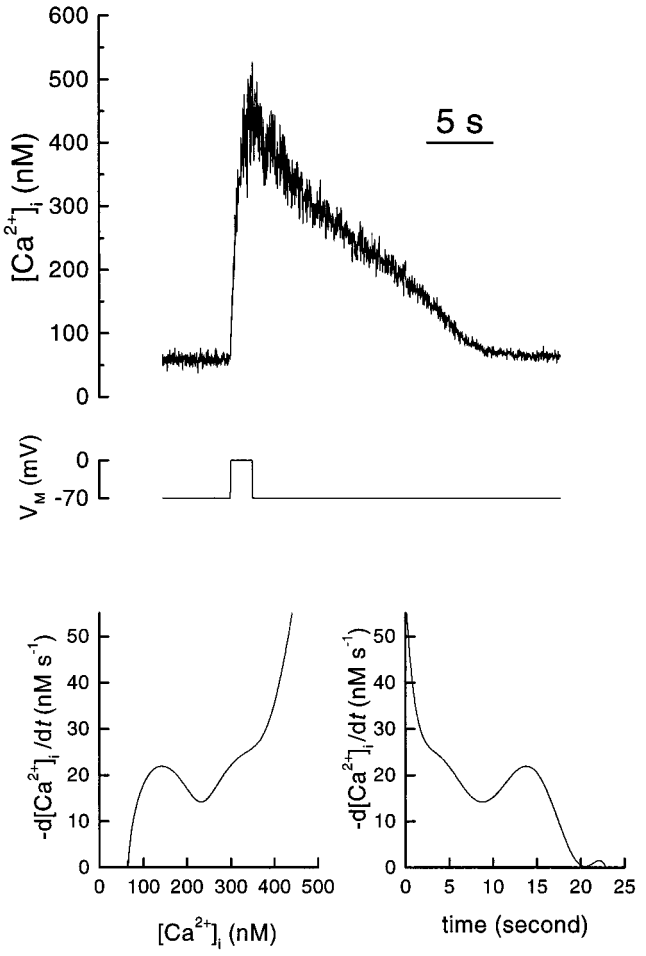

FIGURE 1 Depolarization-evoked increases in $[Ca^{2+}]_i$ and rates of Ca^{2+} removal. A depolarizing pulse to 0 mV from a holding potential of -70 mV (*middle trace*) triggered an increase in $[Ca^{2+}]_i$ (*upper trace*). The elevated $[Ca^{2+}]_i$ returned to the resting level after the termination of the pulse. The declining phase of the Ca^{2+} transient was fitted to a high-order polynomial, and the rate of Ca^{2+} removal was determined from the negative derivative of the fit. The Ca^{2+} removal rate was expressed either as a function of measured $[Ca^{2+}]_i$ (*lower left-hand panel*) or time, where time = 0 is the first data point after repolarization (*lower right-hand panel*). The Ca^{2+} removal profile consisted of three phases. The initial fast phase of Ca^{2+} decline (first phase) was followed by a plateau (second phase). Just before $[Ca^{2+}]_i$ returned to the basal level, the Ca^{2+} removal rate increased. This third phase appears as an upward hump in the lower panels.

RESULTS

Ca^{2+} removal in control cells

Fig. 1 illustrates a typical Ca^{2+} transient (*upper trace*) triggered by a depolarizing pulse to 0 mV from -70 mV (*middle trace*) under control conditions. $[Ca^{2+}]_i$ returned to baseline on repolarization to -70 mV. The decay of the

Ca^{2+} transient displayed a characteristic three-phase pattern (McGeown et al., 1996). After repolarization, Ca^{2+} removal was initially fast (first phase), then slowed (second phase), but before $[Ca^{2+}]_i$ was restored to the basal level, the removal rate again accelerated (third phase). When plotted as a function of $[Ca^{2+}]_i$ (Fig. 1, *lower left panel*) or time (Fig. 1, *lower right panel*), the third phase is apparent as an upward hump.

Thapsigargin and ryanodine inhibit third-phase Ca^{2+} removal

The role of internal stores in the decline of $[Ca^{2+}]_i$ was determined by using the store uptake inhibitor thapsigargin (Thastrup et al., 1990). However, at concentrations over 200 nM, thapsigargin also inhibits the plasma membrane Ca^{2+} current (Rossier et al., 1993; Shmigol et al., 1995) and blocks Ca^{2+}-induced Ca^{2+} release (Kamishima and McCarron, 1997). Therefore, in the presence of thapsigargin, the depolarization-evoked Ca^{2+} transient would be reduced substantially. To overcome this problem, 0.5 μM Bay K 8644 was included in the bathing solution to enhance the opening of voltage-dependent Ca^{2+} channels, thus allowing comparison of Ca^{2+} transients of similar magnitudes. (Bay K 8644 0.5 μM in the absence of thapsigargin did not alter the profile of Ca^{2+} removal ($n = 3$).) Fig. 2 depicts one such experiment. Depolarization to 0 mV (*middle trace*) increased $[Ca^{2+}]_i$ (*upper trace*). $[Ca^{2+}]_i$ sharply declined immediately after repolarization. However, $[Ca^{2+}]_i$ declined without the acceleration of Ca^{2+} removal before returning to the baseline (i.e., phase three was absent). Indeed, the calculated removal rate, shown in the lower panels (Fig. 2), supports this suggestion. To compare the third phase, the Ca^{2+} removal rate was measured at the peak of the third phase. Both thapsigargin (500 nM) and ryanodine (30 μM; a plant alkaloid that should "short circuit" internal stores by locking the release channels in a subconductance state; Smith et al., 1988) significantly ($p < 0.05$) slowed the peak rate of decline in the third phase. Thus the control rate was 28.6 $\pm$ 3.4 nM s^{-1} ($n = 14$), whereas the rate in the presence of thapsigargin was 6.4 $\pm$ 3.1 nM s^{-1} ($n = 5$). The averaged peak third-phase removal rate seen when ryanodine was included in the patch pipette filling solution was also significantly ($p < 0.05$) reduced from control rates (7.7 $\pm$ 2.7 nM s^{-1}; $n = 14$). These results indicate that Ca^{2+} sequestration by the SR contributes to the third phase of removal.

Ryanodine and thapsigargin do not affect first-phase Ca^{2+} removal

To examine the contribution of internal stores to the first phase of Ca^{2+} removal, the time required for the transient to fall by 25% was measured. This measure was preferred to the peak rate of decline in phase 1, because, in some cells, I_{Ca} was not completely inactivated by the end of the pulse.

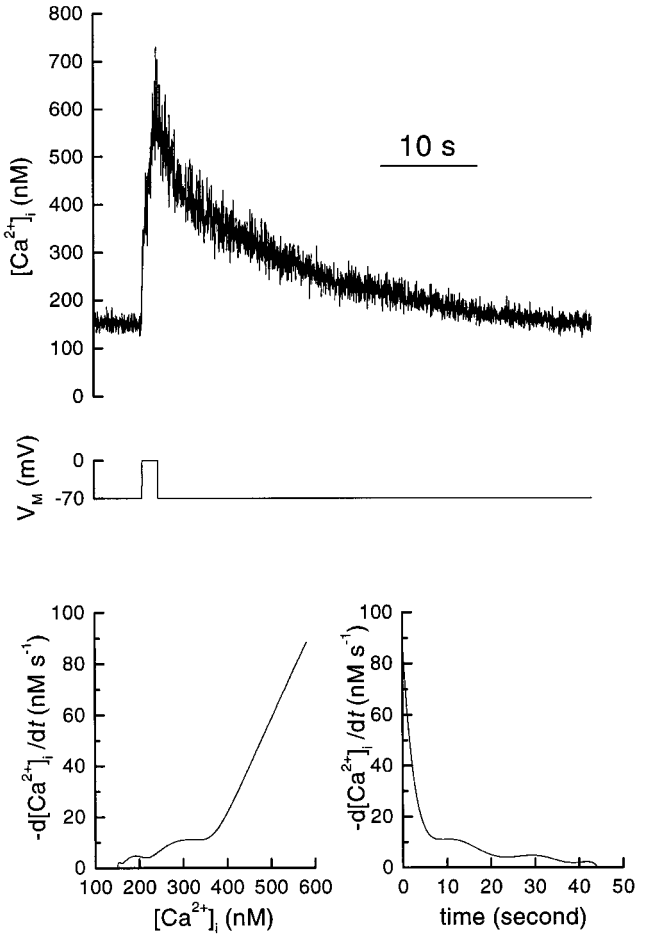

FIGURE 2 Ca^{2+} removal after inhibition of Ca^{2+} store uptake by thapsigargin (500 nM). The third phase (*hump*; see Fig. 1) is no longer present (*lower panels*). Whereas phase 1 of removal is still apparent, the third phase is not. The experiment was carried out in the presence of 0.5 μM Bay K 8644 to produce a Ca^{2+} transient whose magnitude matches that of control cells.

This resulted in a brief suppression of first-phase Ca^{2+} removal rate because of a "Ca^{2+} tail" produced by a tail current. Although the contamination of the Ca^{2+} tail was brief, because I_{Ca} rapidly deactivates at -70 mV, it compromised the accurate detection of the peak first-phase Ca^{2+} removal rate occurring at the instant of repolarization. Therefore, the first phase of removal was summarized by using the time needed for the transient to decrease by 25% ($t_{0.25}$; Fig. 3, *inset*). For example, if $[Ca^{2+}]_i$ increased from a resting value of 100 nM to 700 nM, then $t_{0.25}$ is the time required to reach a $[Ca^{2+}]_i$ of 550 nM. This measure reflected phase one of removal, but was sufficiently far from the time of repolarization to avoid complication of the analysis of $[Ca^{2+}]_i$ decline by the Ca^{2+} tail. Measurements from thapsigargin-treated and ryanodine-treated cells were combined, because each treatment yielded a similar inhibition of the peak third phase removal rate. Fig. 3 (*upper panel*) depicts the average $t_{0.25}$ for controls and thapsigargin or ryanodine-treated cells. The average $t_{0.25}$ for control cells was 3.4 $\pm$ 0.6 s ($n = 14$) and was not significantly different

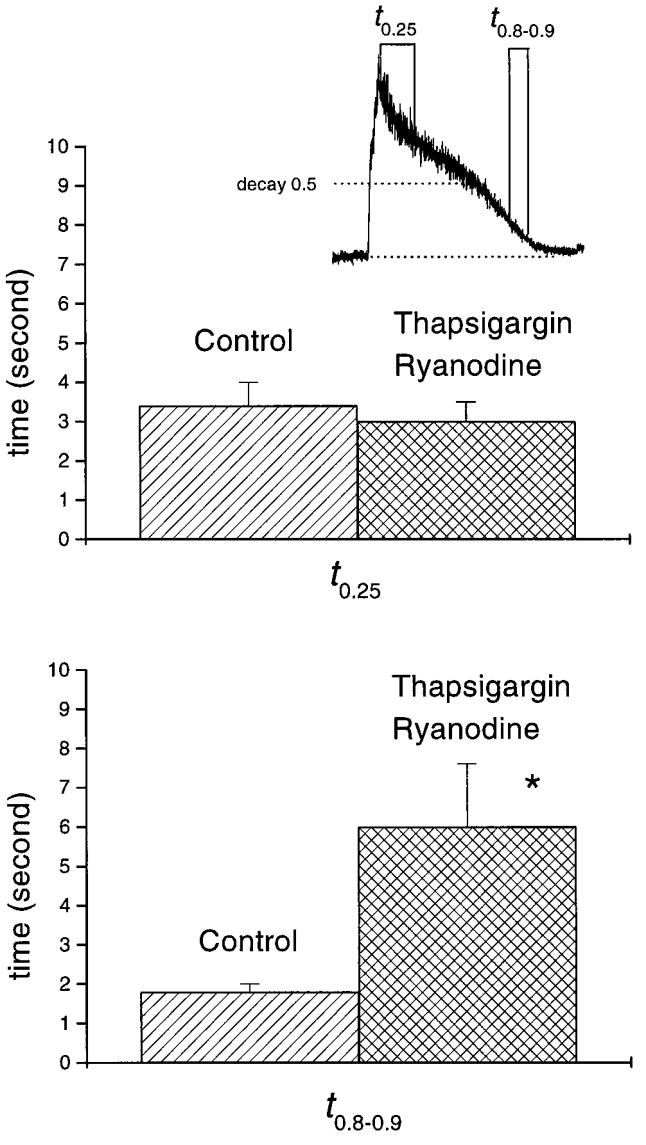

FIGURE 3 Comparison of $t_{0.25}$ and $t_{0.8-0.9}$ in the absence and presence of thapsigargin or ryanodine. The inhibition of Ca^{2+} uptake by sarcoplasmic reticulum did not affect $t_{0.25}$, but significantly prolonged $t_{0.8-0.9}$. The inset illustrates $t_{0.25}$ and $t_{0.8-0.9}$.

from the thapsigargin- or ryanodine-treated cells (3.0 ± 0.5 s, $n = 17$). Thus Ca^{2+} uptake by the SR does not contribute to the first phase of removal.

To determine whether measurement of time ($t_{0.25}$) was sufficiently sensitive to detect alterations in removal rates, the effect of store disruption on removal times during phase 3 was also examined. In this case, the time lapsing between 80% and 90% of the transient decay ($t_{0.8-0.9}$) was used (Fig. 3. *inset*, and in example above, time required for $[Ca^{2+}]_i$ to fall from 220 nM to 160 nM). The rate of decline was significantly slowed during phase 3, as determined by using $t_{0.8-0.9}$ as a measurement of Ca^{2+} removal. Thus the control value was 1.8 ± 0.2 s, whereas the time after thapsigargin or ryanodine treatment was 6.0 ± 1.6 s (Fig. 3, *lower panel*). Because $t_{0.8-0.9}$ appears to detect the inhibition of the third phase by store disruption, $t_{0.25}$ may reasonably reflect first-phase Ca^{2+} removal.

Summary of Ca^{2+} uptake by the SR

The contribution of Ca^{2+} uptake by the SR was examined as a function of $[Ca^{2+}]_i$ (Fig. 4). The average Ca^{2+} removal rate for controls ($n = 14$) was significantly faster than that for thapsigargin- or ryanodine-treated cells ($n = 17$) up to 350 nM $[Ca^{2+}]_i$, supporting the proposal that the SR Ca^{2+} pump is a high-affinity Ca^{2+} clearance mechanism (Kargacin and Kargacin, 1995).

Na^+ dependence of $[Ca^{2+}]_i$ decline

The low-affinity Na^+-Ca^{2+} exchanger is an important Ca^{2+} removal mechanism in some (McCarron et al., 1994; McGeown et al., 1996) but not in other (Ganitkevich and Isenberg, 1991; Fleischmann et al., 1996) smooth muscle cells. Ca^{2+} removal through the Na^+-Ca^{2+} exchanger requires extracellular Na^+, so to examine the exchanger's contribution to $[Ca^{2+}]_i$ decline, particularly during phase 1, extracellular Na^+ was replaced with choline$^+$ (Fig. 5). Depolarization to 0 mV (*middle trace*) evoked a Ca^{2+} transient (*upper trace*). The overall profile of the Ca^{2+} transient was virtually indistinguishable from that of the control cells (Fig. 1), suggesting that the Na^+-Ca^{2+} exchanger does not significantly contribute to the Ca^{2+} removal process. When the Ca^{2+} removal rate was expressed as a function of $[Ca^{2+}]_i$ or time, all three phases were still clearly evident (Fig. 5, *lower panels*). Indeed, the average peak third phase Ca^{2+} removal rate in the presence of

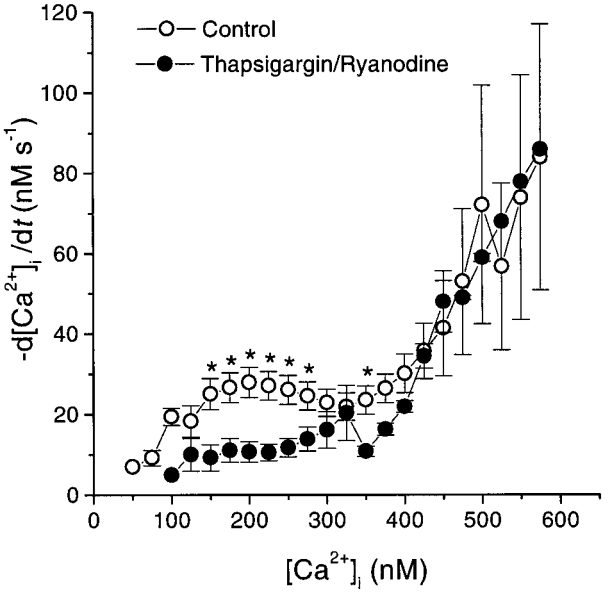

FIGURE 4 Comparison of the rate of Ca^{2+} removal as a function of $[Ca^{2+}]_i$, in the absence or presence of thapsigargin or ryanodine. The third phase removal rate was significantly ($p < 0.05$) faster in control cells than in those treated with store Ca^{2+} uptake inhibitors, as shown by *.

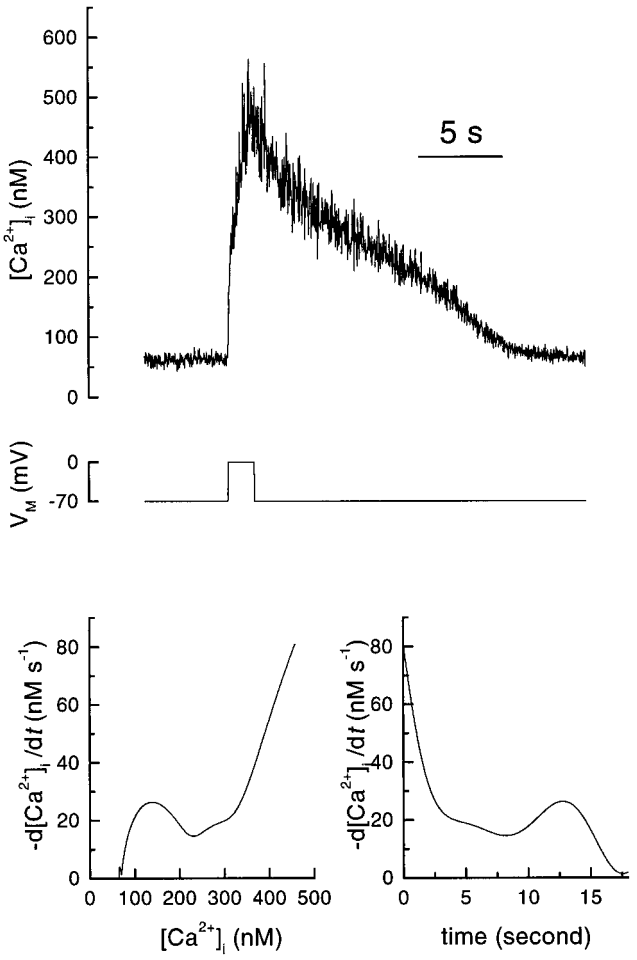

FIGURE 5 Depolarization-evoked Ca^{2+} increases and the rate of $[Ca^{2+}]_i$ after substitution of Na^+ with choline$^+$. Na^+ removal did not alter the Ca^{2+} removal rate (*lower panels*), and all three phases are present.

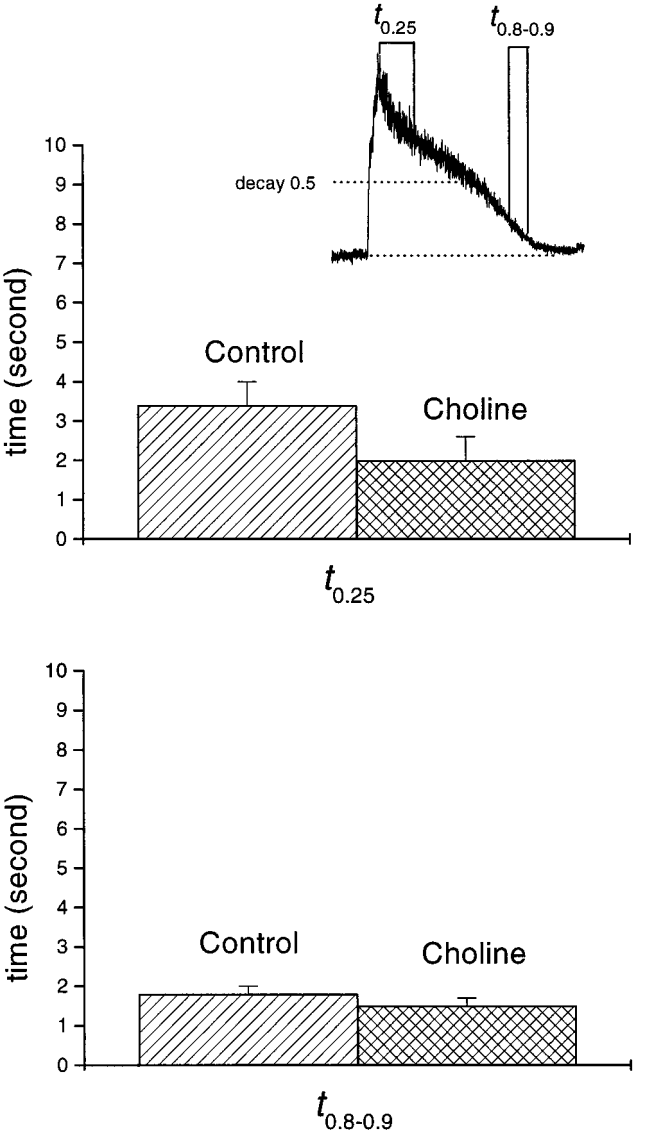

FIGURE 6 Comparison of $t_{0.25}$ and $t_{0.8-0.9}$ in the Na^+-containing (*control*) and Na^+-free choline$^+$-bathed cells. The inhibition of the Na^+-Ca^{2+} exchanger did not affect either $t_{0.25}$ or $t_{0.8-0.9}$. The inset illustrates $t_{0.25}$ and $t_{0.8-0.9}$.

choline$^+$ was 25 ± 5 nM s^{-1} (n = 8), and was not significantly different from the control rates (29 ± 3 nM s^{-1}). Similarly, neither $t_{0.25}$ (2.0 ± 0.6 s, n = 8) nor $t_{0.8-0.9}$ (1.5 ± 0.2 s) was significantly different in the presence or absence of Na^+ (Fig. 6). Thus neither the first phase nor the third phase was affected by Na^+ replacement.

Summary of Ca^{2+} removal in Na^+-free solution

Fig. 7 summarizes the rate of Ca^{2+} removal, in the presence and absence of Na^+, plotted as a function of $[Ca^{2+}]_i$. The average Ca^{2+} removal rate of choline$^+$-bathed solution (n = 8) was not significantly different from that of the control cells (n = 14), supporting the suspicion that Ca^{2+} clearance through the Na^+-Ca^{2+} exchanger is not important in superior artery smooth muscle cells over the $[Ca^{2+}]_i$ range examined.

Summary of Ca^{2+} removal by Ca^{2+} pumps in SR and cell membrane

In rat superior cerebral artery smooth muscle cells, inhibitors of store Ca^{2+} uptake, attenuated the third phase. Therefore, the difference obtained by subtracting the Ca^{2+} removal rate of thapsigargin- or ryanodine-treated cells from the control value should represent Ca^{2+} uptake rate by the SR (Fig. 8). It appears that the store's contribution to Ca^{2+} clearance occurs at low $[Ca^{2+}]_i$. There was no detectable contribution of the Na^+-Ca^{2+} exchanger. By elimination, therefore, the Ca^{2+} removal rates observed in the presence of thapsigargin or ryanodine seem consistent with extrusion by Ca^{2+} pumps in the cell membrane (Fig. 8). It is conceivable, however, that some Ca^{2+} removal processes are time dependent as well as $[Ca^{2+}]_i$ dependent. Hence it is possible that the store Ca^{2+} uptake requires slow up-regulatory mechanisms and is not observed immediately after the repolarization, when $[Ca^{2+}]_i$ is high.

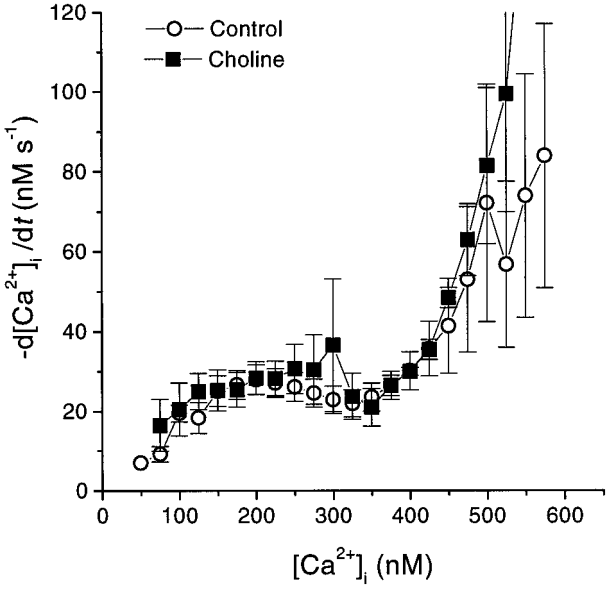

FIGURE 7 Comparison of the Ca^{2+} removal rates expressed as a function of $[Ca^{2+}]_i$ under control conditions and in the presence of choline chloride, a substitute for Na^+. No detectable difference in removal rate occurred.

DISCUSSION

The results provide evidence that both the SR Ca^{2+} pump and the plasma membrane Ca^{2+} pump are important removal mechanisms in resistance artery smooth muscle. The data also indicate that the Na^+-Ca^{2+} exchanger contributes little to the restoration of resting $[Ca^{2+}]_i$ levels after depolarization-evoked increases in $[Ca^{2+}]_i$.

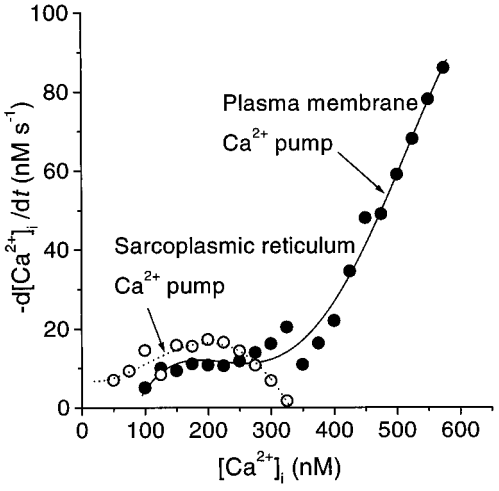

FIGURE 8 A summary of Ca^{2+} removal rate by Ca^{2+} pumps. The removal by the sarcoplasmic reticulum Ca^{2+} pump was determined by subtracting removal rates of thapsigargin- or ryanodine-treated cells from those of controls (*open circles*). The filled circles represent the removal rate of thapsigargin- or ryanodine-treated cells. Because no detectable Ca^{2+} clearance through the Na^+-Ca^{2+} exchanger was detected, Ca^{2+} removal in these cells presumably occurs through Ca^{2+} pumps in the cell membrane.

Ca^{2+} clearance in cardiac myocytes, neurons, and adrenal chromaffin cells has been the subject of detailed investigation (e.g., Balke et al., 1994; Friel and Tsien 1994; Herrington et al., 1996; Babcock et al., 1997). In smooth muscle cells, however, less is known about the Ca^{2+} removal mechanisms. The Ca^{2+} pumps of the internal Ca^{2+} stores have been proposed to have primary responsibility for removing increased $[Ca^{2+}]_i$ after the termination of excitatory stimuli (Kargacin and Kargacin, 1995). However, others have proposed that the Na^+-Ca^{2+} exchanger is physiologically important in smooth muscle cells (e.g., Blaustein et al., 1986; McCarron et al., 1994) or that the sarcolemma Ca^{2+} pump is the main route in clearing Ca^{2+} (e.g., Raemaekers and Wuytack, 1996). Such varying conclusions may be ascribed to differences in tissue, species (Eggermont et al., 1988), or experimental/analytical approach.

Because Ca^{2+}-induced Ca^{2+} release contributed substantially to the depolarization-induced increase in $[Ca^{2+}]_i$ in the resistance artery used in this study (Kamishima and McCarron, 1997), it seemed likely that Ca^{2+} pumps in the stores play an important role in Ca^{2+} removal. Ca^{2+} uptake by the stores was detected as a delayed up-regulation of Ca^{2+} removal (the third phase). The $[Ca^{2+}]_i$ range over which the SR removes Ca^{2+} is consistent with the notion of a high-affinity Ca^{2+} removal mechanism.

The role of Na^+-Ca^{2+} exchange in regulating Ca^{2+} in smooth muscle has been tenaciously debated. Clear evidence from several studies has demonstrated that a Na^+-Ca^{2+} exchanger can substantially alter $[Ca^{2+}]_i$ in smooth muscle, but only when in reverse mode (Ca^{2+} entry mode; e.g., Aaronson and Benham, 1989). Fewer studies have demonstrated that a Na^+-Ca^{2+} exchanger can regulate $[Ca^{2+}]_i$ when operating in forward mode (Ca^{2+} extrusion mode). In the gastric smooth muscle cells of the toad, forward mode Na^+-Ca^{2+} exchanger activity did remove $[Ca^{2+}]_i$ at higher concentrations (>300 nM; McCarron et al., 1994; McGeown et al., 1996). However, in equine tracheal myocytes and guinea pig bladder cells, the Na^+-Ca^{2+} exchanger did not play a significant part in Ca^{2+} clearance from the cytosol (Fleischmann et al., 1996; Ganitkevich and Isenberg, 1991).

In the present study the Na^+-Ca^{2+} exchanger made no detectable contribution to the removal of Ca^{2+}. Thus Na^+ replacement with choline$^+$ did not affect the rate of Ca^{2+} removal over the $[Ca^{2+}]_i$ range tested (Fig. 7). Furthermore, an inward current, which should accompany the rapid decay of Ca^{2+} through Na^+-Ca^{2+} exchanger activity, was not detected (not shown). Finally, Ca^{2+} removal was voltage independent. For example, holding the membrane potential at 120 mV after the voltage pulse to 0 mV should attenuate the rate of Ca^{2+} removal (McCarron et al., 1994). On repolarization to −70 mV from +120 mV, an accelerated rate of Ca^{2+} removal would be expected. However, in three cells in which this high-voltage protocol was tested, the inhibition of Ca^{2+} removal during high voltage was not observed. Indeed, the Ca^{2+} removal rate just before the voltage was changed from +120 mV to −70 mV was

18.7 ± 1.6 nM s^{-1}, not significantly different from that just after the voltage change (18.6 ± 1.1 nM s^{-1}, $n = 3$).

Investigations of Ca^{2+} removal processes have frequently relied on measurements such as muscle relaxation times, $[Ca^{2+}]_i$ decay times, or rate constants of decline. Each of these measurements will almost certainly vary as the $[Ca^{2+}]_i$ range over which the measurement is made changes. Experiments designed to inhibit a removal system, which also independently alter resting $[Ca^{2+}]_i$, could alter rate constants or time measurements regardless of changes in Ca^{2+} removal. Unless they are well controlled, such measurements will distort the contributions of the underlying removal mechanisms. Complicating matters further, when rate constants or time is used to measure decline, is the probability that more than one mechanism is operating in parallel to clear Ca^{2+} from the cytosol. Thus inhibition of one removal pathway will increase the substrate (Ca^{2+}) available to the other systems with a possible increase in their removal rates. Again, this could frustrate the determination of the underlying removal mechanisms. The method of analysis of removal is, therefore, of considerable importance, and velocity plotted against $[Ca^{2+}]_i$, in all probability, is the most reliable.

Together, the results presented highlight the importance of the plasma membrane Ca^{2+} pump and SR Ca^{2+} pump in regulating $[Ca^{2+}]_i$ in smooth muscle and support the idea that Na^+-Ca^{2+} exchange may contribute little to Ca^{2+} removal in mammalian smooth muscle.

This project was funded by The Wellcome Trust (036885/Z/92/Z).

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