

The buffer barrier hypothesis, $[Ca^{2+}]_i$ homogeneity, and sarcoplasmic reticulum function in swine carotid artery

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1. The goal of this study was to evaluate the buffer barrier hypothesis in an intact arterial smooth muscle. Specifically, we investigated the interrelationships between intracellular $[Ca^{2+}]_i$ homogeneity and sarcoplasmic reticulum function in swine carotid artery.
2. We measured focal changes in $[Ca^{2+}]_i$ by exploiting the different characteristics of several $[Ca^{2+}]_i$ indicators: (1) aequorin, which can detect focal increases in $[Ca^{2+}]_i$ such as those that occur in the subplasmalemmal region ($[Ca^{2+}]_{pm}$); (2) fura-2, which is primarily a measure of mean cytoplasmic $[Ca^{2+}]_c$; and (3) force, which reflects increases in $[Ca^{2+}]_i$ near the contractile apparatus. We then estimated the relative degree of $[Ca^{2+}]_i$ homogeneity with the aequorin/fura-2 ratio. Finally, we inhibited sarcoplasmic reticulum Ca^{2+} pumping with cyclopiazonic acid (CPA), an inhibitor of the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA).
3. We found that, after Ca^{2+} depletion, the sarcoplasmic reticulum could be partially reloaded with Ca^{2+} by manipulations that increased the aequorin signal relatively more than the fura-2 signal. Complete reloading required large increases in the fura-2 signal. These data suggest that increases in $[Ca^{2+}]_{pm}$ (as measured with aequorin) can partially reload the sarcoplasmic reticulum, but complete reloading required increases in $[Ca^{2+}]_c$ (as measured with fura-2). Reloading could be partially inhibited by 10 μM CPA, indicating that SERCA function was important for reloading.
4. In unstimulated arteries, 10 μM CPA increased the fura-2 signal without altering the aequorin signal, thereby decreasing the aequorin/fura-2 ratio. Removal of extracellular Ca^{2+} without CPA also reduced the aequorin/fura-2 ratio. These data suggest that resting cells have a $[Ca^{2+}]_i$ gradient with $[Ca^{2+}]_{pm} > [Ca^{2+}]_c$; this gradient is maintained by SERCA function.
5. CPA slowed the decline in the fura-2 signal observed when histamine stimulation was removed. This result is consistent with the concept of vectorial Ca^{2+} efflux in which Ca^{2+} pumping by SERCA reduces $[Ca^{2+}]_c$ after stimulation.
6. Ca^{2+} depletion by prior treatment with 100 μM histamine and CPA transiently attenuated subsequent histamine-induced aequorin and fura-2 transients. The effect on contraction was smaller: a delay in contraction of approximately 10 s. These data suggest that histamine-induced Ca^{2+} release has at least a small role in the initial phase of contraction; however, other contractile mechanisms appear to be able to compensate for loss of Ca^{2+} release with only modest changes in contraction kinetics.
7. These data suggest that there is a complex interrelationship between smooth muscle sarcoplasmic reticulum function and $[Ca^{2+}]_i$ in at least two cytoplasmic compartments. $[Ca^{2+}]_{pm}$ and $[Ca^{2+}]_c$ can differentially regulate sarcoplasmic reticulum Ca^{2+} filling; and sarcoplasmic reticulum function regulates $[Ca^{2+}]_{pm}$ and $[Ca^{2+}]_c$.

It is well accepted that contractile agonist stimulation of smooth muscle induces Ca^{2+} release from a non-mitochondrial intracellular store (reviewed in Rembold, 1996). This store has been identified as the sarcoplasmic reticulum. 1,4,5-Inositol trisphosphate (1,4,5-IP₃) is proposed to be the primary second messenger responsible for Ca^{2+} release. The sarcoplasmic reticulum also contains a Ca^{2+} pump, termed the sarco(endo)plasmic reticulum ATPase (SERCA), which appears to be primarily responsible for Ca^{2+} uptake into the sarcoplasmic reticulum. These data suggest that smooth muscle contains the necessary proteins for agonist stimulated Ca^{2+} release. However, the relative importance of Ca^{2+} release in the resultant contraction has yet to be determined. Additionally, it is possible that the sarcoplasmic reticulum has role(s) in the regulation of intracellular $[\text{Ca}^{2+}]_i$ ($[\text{Ca}^{2+}]_i$) beyond agonist-induced Ca^{2+} release.

Smooth muscle sarcoplasmic reticulum is a complex three-dimensional tubular structure in the cytosol. Electron microscopy reveals the existence of a portion of the sarcoplasmic reticulum closely apposed near the plasma membrane (Devine *et al.* 1972). This region of close apposition may have a special role in regulation of $[\text{Ca}^{2+}]_i$ because a number of Ca^{2+} transport proteins, such as the Na^+ - Ca^{2+} exchanger, are preferentially localized to this region (Villa *et al.* 1993; Moore *et al.* 1993). It is possible that Ca^{2+} flux through this region may be involved in Ca^{2+} influx or efflux. For example, Ca^{2+} influx across the plasma membrane may be important to refill the sarcoplasmic reticulum after Ca^{2+} release. Ca^{2+} could also flow in a reverse direction. When contractile stimuli are removed, the sarcoplasmic reticulum may pump Ca^{2+} into its lumen, transport it to near the plasma membrane, and release it into the subsarcolemmal cytoplasm so it can be exported into the extracellular space.

This specialized region of cytoplasm between the sarcoplasmic reticulum and the plasma membrane may have a different $[\text{Ca}^{2+}]$ than the remaining cytoplasm. In 1977, Van Breemen hypothesized that there are functional $[\text{Ca}^{2+}]_i$ domains inside smooth muscle cells: (1) a contractile domain encompassing most of the cytoplasm (hereafter termed $[\text{Ca}^{2+}]_c$), and (2) a subplasmalemmal domain (hereafter termed $[\text{Ca}^{2+}]_{pm}$) (Van Breemen, 1977; Van Breemen *et al.* 1986). The latter domain includes the region where the sarcoplasmic reticulum is closely apposed to the plasma membrane. According to this hypothesis, the $[\text{Ca}^{2+}]_{pm}$ may, at times, be higher than $[\text{Ca}^{2+}]_i$. He also hypothesized that higher $[\text{Ca}^{2+}]_{pm}$ may have a role in refilling the sarcoplasmic reticulum with Ca^{2+} . Subsequently other investigators, working in other cell types, have hypothesized the existence of subplasmalemmal Ca^{2+} domains (Nagai *et al.* 1989; Rembold, 1989; Takemura & Putney, 1989; Lederer *et al.* 1990; Augustine & Neher, 1992). Evidence for focal increases in $[\text{Ca}^{2+}]_i$ was inferred by comparing fura-2-estimated $[\text{Ca}^{2+}]_i$ with $[\text{Ca}^{2+}]_{pm}$ as measured with calcium-activated K^+ (K_{Ca}) channel activity (Stehno-Bittel & Sturek, 1992; Yoshikawa *et al.* 1996), synaptic vesicle fusion (Smith &

Augustine, 1988; Augustine & Neher, 1992; Brose *et al.* 1992), or Ca^{2+} dependent Cl^- currents (Osipchuk *et al.* 1990) in isolated patch clamped cells. Unfortunately, measurement of $[\text{Ca}^{2+}]_{pm}$ with fluorescent dyes has been difficult because (1) the region is very small and (2) the fluorescent dyes, *per se*, act as mobile Ca^{2+} buffers which attenuate and change the spatial characteristics of the Ca^{2+} domains (Neher & Augustine, 1992).

We recently developed a method to estimate whether cellular $[\text{Ca}^{2+}]$ is homogeneous (e.g. $[\text{Ca}^{2+}]_{pm} = [\text{Ca}^{2+}]_c$) or inhomogeneous (i.e. $[\text{Ca}^{2+}]_{pm} > [\text{Ca}^{2+}]_c$) in the cell. We measure $[\text{Ca}^{2+}]_i$ with both aequorin and fura-2 (Rembold *et al.* 1995). This dual measurement allows estimation of relative $[\text{Ca}^{2+}]_i$ homogeneity based on the characteristics of these two Ca^{2+} indicators (Yue & Wier, 1998). Aequorin is a Ca^{2+} sensitive protein that is very sensitive to increases in $[\text{Ca}^{2+}]$ that occur in small regions of the cell (e.g. $[\text{Ca}^{2+}]_{pm}$). In contrast, fura-2 is predominantly a measure of mean $[\text{Ca}^{2+}]$ (i.e. $[\text{Ca}^{2+}]_c$). The ratio of the observed aequorin and fura-2 signals is an index of the relative degree of $[\text{Ca}^{2+}]_i$ homogeneity in intact swine carotid arterial tissues (Rembold *et al.* 1995). Increases in this ratio suggest a more inhomogeneous $[\text{Ca}^{2+}]$ distribution.

The goal of this study was to evaluate the physiological roles of $[\text{Ca}^{2+}]_i$ homogeneity and sarcoplasmic reticulum function in the regulation of arterial smooth muscle $[\text{Ca}^{2+}]_i$ and contraction. In this study we tested four hypotheses: (1) that increases in $[\text{Ca}^{2+}]_{pm}$ were primarily responsible for refilling of the sarcoplasmic reticulum with Ca^{2+} ; (2) that the sarcoplasmic reticulum maintains a $[\text{Ca}^{2+}]$ gradient within unstimulated smooth muscle cells, specifically that basal levels of Ca^{2+} influx increase $[\text{Ca}^{2+}]_{pm}$ more than $[\text{Ca}^{2+}]_c$ in unstimulated smooth muscle; (3) that the sarcoplasmic reticulum is important in removal of Ca^{2+} from the centre of the cell; and (4) that Ca^{2+} release from the sarcoplasmic reticulum is crucial for the initial phase of agonist-induced contractions.

METHODS

Swine common carotid arteries were obtained from an abattoir (Smithfield Co., Smithfield, VA, USA), dissected, mounted for isometric stress measurement, and the optimal length for stress development determined (Rembold & Murphy, 1988). The physiological saline solution (PSS) for arterial tissues contained (mM): NaCl, 140; KCl, 4.7; Mops, 2; CaCl_2 , 1.6; MgCl_2 , 1.2; Na_2HPO_4 , 1.2; EGTA, 0.02; D-glucose, 5.6; pH adjusted to 7.4 at 37 °C.

Aequorin-estimated $[\text{Ca}^{2+}]$

$[\text{Ca}^{2+}]$ was estimated in one set of tissues with the photoprotein aequorin loaded intracellularly by reversible hyperpermeabilization (Rembold & Murphy, 1988). The aequorin-derived light was collected with a photomultiplier tube and the photon count per second (L) was divided by an estimate of the total [aequorin] (L_{max}). The logarithm of this ratio ($\log L/L_{max}$) depends on $[\text{Ca}^{2+}]$. Light signals are reported as a change in $\log L/L_{max}$ in which the

resting $\log L/L_{\max}$ is subtracted from all subsequent $\log L/L_{\max}$ values. This normalization markedly decreased interexperimental variability and provided enhanced sensitivity to small changes in [Ca²⁺]_i (Gilbert *et al.* 1991). Aequorin light signals were calibrated in Ca²⁺-EGTA buffers at 37 °C with [Mg²⁺]_i = 0.5 mM (Gilbert *et al.* 1991). We do not report estimated [Ca²⁺]_i with each experiment because assumptions introduce errors. For example, [Mg²⁺]_i affects aequorin calibration substantially (Blinks *et al.* 1982). We present aequorin data as the change in $\log L/L_{\max}$. See our prior publication (Rembold *et al.* 1995) for discussion of aequorin localization and focal aequorin depletion.

Fura-2-estimated intracellular [Ca²⁺]_i

[Ca²⁺]_i was estimated in a second set of intact swine carotid medial tissues with fura-2 loaded intracellularly by incubation in 20 μM of the acetylmethoxy ester of fura-2 (fura-2 AM) for 3–5 h at 37 °C (Gilbert *et al.* 1991). The final loading solution was PSS containing 20 μM fura-2 AM and 250 μM neostigmine to inhibit breakdown of fura-2 AM by extracellular esterases. After loading, arterial strips were washed in PSS for 30–45 min before each experiment. Tissues were mounted isometrically and stretched to ~1.05 of the optimal length for force development to decrease movement artifacts (Gilbert *et al.* 1991). The fura-2-loaded tissues were sequentially excited with 340 ± 5, 360 ± 5 and 380 ± 5 nm light with a rotating filter wheel system. The emission light was measured at 525 ± 25 nm with a photomultiplier tube. The fluorescence signals were electronically demultiplexed and the fura-2 340, 360, and 380 fluorescence signals and the force signals were converted to digital signals and stored on a personal computer. After completing pharmacological manipulations, incubation in 5 mM MnCl₂ lysed the cells, quenched fura-2 fluorescence and allowed measurement of background fluorescence subtracted from all fluorescence measurements. We do not report estimated [Ca²⁺]_i with each experiment because assumptions introduce errors. For example, protein binding, extracellular leakage, and loading of fura-2 into organelles affects fura-2 calibration (Uto *et al.* 1991; Shuttleworth & Thompson, 1991). We present fura-2 data as a background-subtracted 340/380 ratio. See our prior publication (Rembold *et al.* 1995) for discussion of fura-2 localization and other artifacts of fura-2.

Estimation of [Ca²⁺]_i homogeneity with the aequorin/fura-2 ratio

Aequorin and fura-2 detect [Ca²⁺]_i differently. Fura-2 has one Ca²⁺ binding site and alterations in [Ca²⁺]_i cause the fluorescence signal to vary over approximately one order of magnitude (Gryniewicz *et al.* 1985). Aequorin has three Ca²⁺ binding sites (it is in the EF-hand superfamily of Ca²⁺ binding proteins) and changes in [Ca²⁺]_i regulate aequorin light emission over six orders of magnitude (Blinks *et al.* 1982). The slope of the aequorin calibration (~2.5) is steeper than the slope of the fura-2 calibration (~1). Therefore, absolute aequorin signals increase much more than fura-2 signals in response to given increases in [Ca²⁺]_i. If [Ca²⁺]_i is homogeneous, both aequorin and fura-2 give accurate representations of mean [Ca²⁺]_i; however, if [Ca²⁺]_i is not uniform, aequorin and fura-2 will report different values for mean [Ca²⁺]_i.

To demonstrate this phenomenon, suppose there is a cell that has equally distributed aequorin and fura-2 in the cytoplasm. This hypothetical cell also has two regions with different [Ca²⁺]_i: (1) [Ca²⁺]_c, the [Ca²⁺]_i in the centre of the cell, which equals 100 nM, and (2) [Ca²⁺]_{pm}, the [Ca²⁺]_i near the plasma membrane, which equals 1000 nM. Within each region, [Ca²⁺]_i is uniform. Given these levels of [Ca²⁺]_i in each region, the resulting focal aequorin and fura-2 signal from each region can be predicted. The sum of the

focal signals resulting from both regions can be calculated depending on the relative volume of the two regions. If the volume of the subplasmalemmal region is 10% of the cell volume, then the measured aequorin signal would predict a mean [Ca²⁺]_i of 522 nM, the measured fura-2 signal would predict a mean [Ca²⁺]_i of 166 nM, and the actual mean [Ca²⁺]_i would be 190 nM. Under these conditions, neither indicator was truly representative: aequorin overestimated and fura-2 underestimated true mean [Ca²⁺]_i, a reflection of the non-linearity of the calibration curves of both indicators.

We can exploit the different predictions of mean global [Ca²⁺]_i observed with aequorin and fura-2 as a measure of [Ca²⁺]_i homogeneity. The ratio of the two Ca²⁺ indicators (aequorin/fura-2) depends on the relative volume of the two regions and the [Ca²⁺]_i within each region. In this study, we calculated the ratio of the aequorin signal to fura-2 signal as a qualitative and relative index of [Ca²⁺]_i homogeneity. Unfortunately, we cannot convert calculated ratios from experiments into estimates of both [Ca²⁺]_i and the relative volume of these two regions. Calculation of both would require another measure of homogeneity. Furthermore, real cells will have varying levels of [Ca²⁺]_i in multiple, not just two, regions. See our prior publication for further discussion of these calculations and predictions (Rembold *et al.* 1995).

The aequorin/fura-2 ratio formula was empirically derived (Rembold *et al.* 1995). We made the assumption that [Ca²⁺]_i was the most homogeneous when the tissue was either (1) bathed in PSS with no extracellular Ca²⁺ or (2) maximally stimulated with histamine in the presence of extracellular Ca²⁺. In the absence of extracellular Ca²⁺ there would be no Ca²⁺ influx, and therefore possibilities for focal increases in Ca²⁺ would be minimized at a low mean [Ca²⁺]_i. During sustained histamine stimulation, activation (i.e. myosin phosphorylation and force) is maximal despite modest increases in [Ca²⁺]_i. In typical experiments, we find the fura-2 signal in zero Ca²⁺ is ~1.0 and it increases to ~2.2 with 10 min of maximal histamine stimulation. Under the same conditions, the aequorin signal in zero Ca²⁺ is ~-0.2 and increases to a sustained value of ~0.4. Given these values, we calculated the aequorin/fura-2 ratio from mean aequorin and fura-2 data in the top two panels with the following formula:

$$\text{Ratio} = ((\text{aequorin} + 0.2) \cdot 2 + 1) / \text{fura}2.$$

The resulting corrected aequorin/fura-2 ratio was 1.0 both in zero Ca²⁺ and with maximal histamine stimulation. Increases in this ratio represent a relatively less homogeneous Ca²⁺ distribution. Supporting our original assumptions was the finding that observed aequorin/fura-2 ratios never significantly decreased below 1.0. In the presence of homogeneous [Ca²⁺]_i, the calculated aequorin/fura-2 ratio remains relatively independent of [Ca²⁺]_i at [Ca²⁺]_i above 50 nM. Below 50 nM [Ca²⁺]_i the aequorin/fura-2 ratio depended on [Ca²⁺]_i (specifically it decreased with lower [Ca²⁺]_i). Prior studies revealed that histamine or 20–40 mM [K⁺]_o did not alter the aequorin/fura-2 ratio, suggesting that stimuli that primarily increase global [Ca²⁺]_i do not alter the aequorin/fura-2 ratio (Rembold *et al.* 1995). Prior studies also revealed that loading swine carotid artery with both aequorin and fura-2 did not alter the aequorin or fura-2 signal in response to histamine or high [K⁺]_o (Gilbert *et al.* 1991).

Fura-2-estimated Mn²⁺ influx

Ca²⁺ influx was estimated in a third set of intact swine carotid medial tissues loaded with fura-2 as above. The 360 nm fluorescence was collected and normalized such that fluorescence before addition

of Mn^{2+} to the bathing solution was 1.0 and the minimal fluorescence after lysis with 5 mM $MnCl_2$ was 0.0. Mn^{2+} influx rates were calculated from the slope of the linear regression line of the 360 nm signal measured at 1 s intervals from 10 to 130 s after addition of 0.5 mM Mn^{2+} (Chen & Rembold, 1992).

Chemicals. Aequorin was obtained from John Blinks (Friday Harbor Laboratories, Friday Harbor, WA, USA). Fura-2 AM was obtained from Molecular Probes. Histamine and other chemicals were obtained from Sigma.

RESULTS

Mechanisms for refilling of the sarcoplasmic reticulum with Ca^{2+}

Our first goal was to evaluate the sources of Ca^{2+} responsible for Ca^{2+} refilling of the sarcoplasmic reticulum after Ca^{2+} depletion. We hypothesized that increases in $[Ca^{2+}]_{pm}$ were necessary for refilling of the sarcoplasmic reticulum with Ca^{2+} . Figure 1 shows the basic protocol. At 10 min,

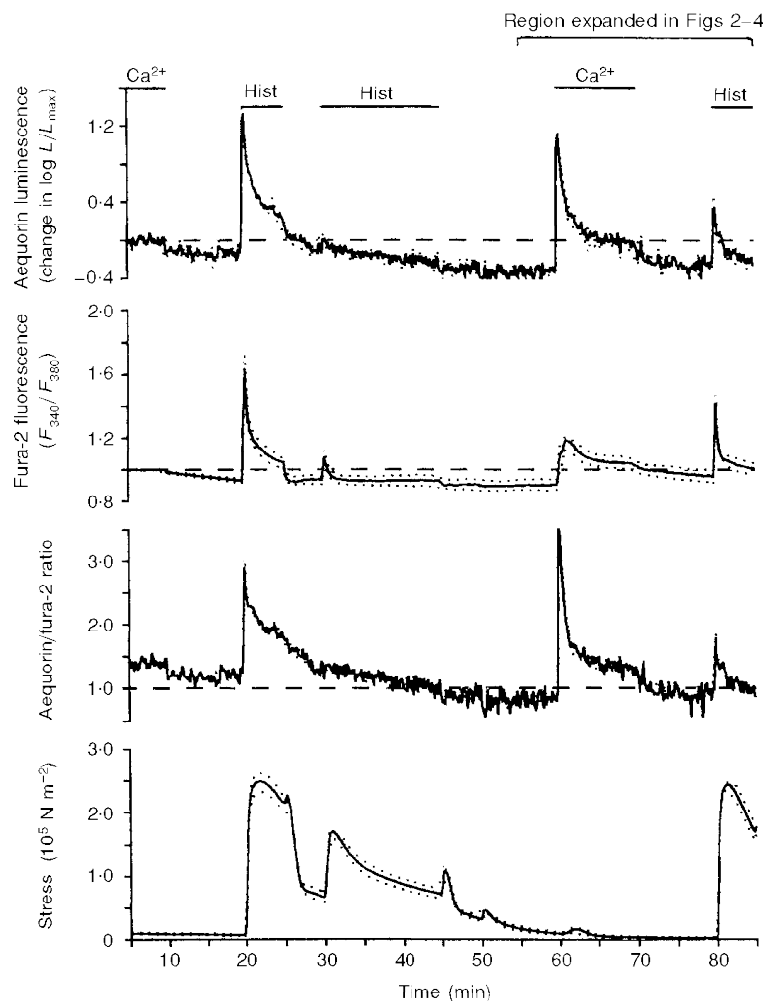


Figure 1. Demonstration of the protocol for studying the mechanism for refilling of the sarcoplasmic reticulum with Ca^{2+}

Time courses are shown for aequorin-estimated $[Ca^{2+}]_i$ (change in $\log L/L_{max}$, top panel), fura-2-estimated $[Ca^{2+}]_i$ (background corrected 340/380 fluorescence ratio, second panel), the aequorin/fura-2 ratio (third panel), and active stress ($\times 10^5 \text{ N m}^{-2}$, bottom panel). Data are from for swine carotid medial tissues ($n = 4-6$). Extracellular Ca^{2+} was nominally removed at 10 min by changing the bathing solution to a solution with no added Ca^{2+} . At 20 min, some of the tissues were treated with 10 μM CPA (e.g. Fig. 4, not shown in this experiment), which was then present in all subsequent solutions and other tissues were not treated with CPA. At 20 min, the tissues were stimulated with 100 μM histamine (Hist) for 5 min and the histamine was then washed out. At 30 min, the tissues were stimulated with 100 μM histamine for 15 min and the histamine was then washed out. At 60 min, extracellular Ca^{2+} was restored to the tissues for 10 min. In some experiments this solution contained other activating agents. At 70 min, extracellular Ca^{2+} was removed. At 80 min, the tissues were stimulated with 100 μM histamine. The data collected from 55 to 85 min are expanded in the subsequent Figs 2-4. Data were collected at 1 s intervals and averaged over 10 s for plotting. Mean data values are shown as a continuous line and the dotted lines represent ± 1 s.e.m. Only stress from the fura-2 experiments are shown for clarity (the aequorin stress data were similar).

extracellular Ca²⁺ was nominally removed by changing the bathing solution to a solution with no added Ca²⁺. This solution has an extracellular [Ca²⁺]_o of approximately 10 μM caused by Ca²⁺ contamination from the glassware and the other salts in the physiological saline solution. Nominal removal of extracellular Ca²⁺ decreased both the aequorin and fura-2 signals and the aequorin/fura-2 ratio. This decrease in the aequorin/fura-2 ratio is consistent with development of a more homogeneous Ca²⁺ distribution. At 20 min, the tissues were stimulated with 100 μM histamine for 5 min. This induced a large contraction and large increases in the aequorin and fura-2 signals indicating a Ca²⁺ release from the intracellular store. There was an increase in the aequorin/fura-2 ratio consistent with our prior report that histamine stimulation in the absence of extracellular Ca²⁺ induced an inhomogeneous increase in [Ca²⁺]_i (Rembold *et al.* 1995). At 30 min, the tissues were restimulated with 100 μM histamine for 15 min. This induced a modest contraction and a smaller increase in the aequorin and fura-2 signal consistent with the prior partial release of Ca²⁺ from the sarcoplasmic reticulum. At 45 min, histamine was washed out repeatedly. At 60 min, extracellular Ca²⁺ was restored to 1.6 mM for 10 min. Restoration of extracellular Ca²⁺ was associated with a large increase in the aequorin signal and the aequorin/fura-2 ratio. There was a smaller increase in the fura-2 signal and force. These data suggest that the increase in [Ca²⁺]_i occurred only in a focal region of the cell, probably the subplasmalemmal space. After removal of extracellular Ca²⁺, the tissues were for a third time stimulated with 100 μM histamine at 80 min, inducing a contraction and an increase in the aequorin and fura-2 signals. This indicates that the 10 min treatment with extracellular Ca²⁺ partially refilled the sarcoplasmic reticulum with Ca²⁺ allowing subsequent histamine-induced Ca²⁺ release.

To evaluate the sources of Ca²⁺ responsible for Ca²⁺ refilling of the sarcoplasmic reticulum, we altered the conditions present during restoration of extracellular Ca²⁺. The data in Figs 2–4 show only the end of the protocol described in Fig. 1 (specifically the data from 55 to 85 min in Fig. 1). All tissues were treated identically with the exception that the Ca²⁺ restoration solution, present from 60 to 70 min in the protocol, was varied.

First, we compared tissues that had Ca²⁺ restored with (1) no other treatment (i.e. the control shown in Fig. 1), (2) 100 μM histamine, (3) 100 μM histamine plus 109 mM [K⁺]_o, and (4) 109 mM [K⁺]_o without histamine. The presence of histamine during Ca²⁺ restoration inhibited Ca²⁺ refilling of the sarcoplasmic reticulum (Fig. 2, second from the left panel). During Ca²⁺ restoration, 100 μM histamine induced large increases in force and the aequorin and fura-2 signals. After removal of extracellular Ca²⁺, repeat histamine stimulation induced smaller increases in force and the aequorin signal than those observed when Ca²⁺ restoration occurred in the absence of histamine (the control is the left panel in Fig. 2). The fura-2 signal was modestly decreased.

Ca²⁺ restoration in the presence of both 100 μM histamine and 109 mM [K⁺]_o induced force, aequorin, and fura-2 signals similar to those observed with Ca²⁺ restoration in the presence of histamine alone (Fig. 2, second from the right panel). This suggests that histamine, rather than the high [Ca²⁺]_o during refilling, was responsible for the decreased refilling of the sarcoplasmic reticulum.

High [K⁺]_o alone during Ca²⁺ restoration enhanced Ca²⁺ refilling of the sarcoplasmic reticulum. During Ca²⁺ restoration, 109 mM [K⁺]_o alone induced large increases in force and the aequorin and fura-2 signals similar to those observed with histamine or histamine plus 109 mM [K⁺]_o (Fig. 2, right panel). After removal of extracellular Ca²⁺, repeat histamine stimulation induced larger increases in the aequorin and fura-2 signals and in force than those observed when Ca²⁺ restoration occurred in the presence of histamine (either with or without high [K⁺]_o). The aequorin and fura-2 signals observed with histamine stimulation after high [K⁺]_o Ca²⁺ restoration were both larger than the control response. These data suggest that the form of stimulation, rather than high [Ca²⁺]_o *per se*, determined sarcoplasmic reticulum Ca²⁺ refilling. Histamine, in spite of producing higher [Ca²⁺]_o, inhibited sarcoplasmic reticulum Ca²⁺ refilling. One potential explanation is that histamine-dependent increases in [1,4,5-IP₃] could prevent refilling by continued Ca²⁺ release into the cytosol through open IP₃ receptor channels. Another possibility is that the histamine receptor was desensitized by prior histamine stimulation.

We recently found a protocol that increases the aequorin signal to high levels with only modest increases in the fura-2 signal or force. This is consistent with the protocol inducing a focal increase in subplasmalemmal [Ca²⁺]_i ([Ca²⁺]_{pm}) without a large increase in central [Ca²⁺]_i ([Ca²⁺]_o). The experimental protocol involves blocking L-type Ca²⁺ channels with 10 μM diltiazem and substituting Na⁺ with choline⁺ to increase [Ca²⁺]_i primarily via inhibition of Na⁺–Ca²⁺ exchange (Rembold *et al.* 1992). We compared tissues that had Ca²⁺ restored with (1) no other treatment (the control from Fig. 1), (2) diltiazem plus choline⁺ substitution for Na⁺, and (3) choline⁺ substitution for Na⁺ without diltiazem. As previously reported (Van Riper *et al.* 1996), diltiazem plus choline⁺ induced a large increase in the aequorin signal with only modest increases in force and the fura-2 signals (Fig. 3, centre panel). This produced a large increase in the aequorin/fura-2 ratio indicating a primarily focal increase in [Ca²⁺]_i. After removal of extracellular Ca²⁺, histamine stimulation induced a smaller contraction than, but similar increases in the aequorin and fura-2 signals to, those observed in the control Ca²⁺ restoration. These data suggest that the focal increase in [Ca²⁺]_i in the subplasmalemmal region did not enhance sarcoplasmic reticulum Ca²⁺ refilling. Ca²⁺ restoration in the presence of choline⁺ alone induced large increases in both the aequorin and fura-2 signals (Fig. 3, right panel). After removal of extracellular Ca²⁺, repeat histamine stimulation induced a larger contraction and larger increases in the aequorin and fura-2 signals than those

observed in either the control or diltiazem–choline⁺ Ca²⁺ restoration. These data suggest that higher [Ca²⁺]_i in the central cytoplasm (i.e. induced by either choline⁺ or high [K⁺]_o) was required to enhance Ca²⁺ refilling. It is probable that increases in [Ca²⁺]_{pm} are an important mechanism for partially refilling the sarcoplasmic reticulum as stated in the first hypothesis. However, complete filling of the sarcoplasmic reticulum required increases in [Ca²⁺]_c.

Next, we evaluated the role of SERCA, the Ca²⁺ pump located in the sarcoplasmic reticulum membrane, in refilling of the sarcoplasmic reticulum with Ca²⁺. Tissues were treated as in Fig. 3 with the exception that cyclopiazonic acid (CPA),

a water soluble specific inhibitor of SERCA, was added with the first addition of histamine and was present throughout the remaining protocol (i.e. from 20 to 85 min in Fig. 1). Restoration of extracellular Ca²⁺ alone was associated with large increases in the aequorin signal and the aequorin/fura-2 ratio (Fig. 4, left panel). There was a larger increase in the fura-2 signal and force than that observed in the absence of CPA (cf. left panels in Figs 3 and 4). After removal of extracellular Ca²⁺, stimulation with 100 μM histamine induced a small contraction associated with no change in the aequorin or fura-2 signals. This indicates that 10 μM CPA prevented sarcoplasmic reticulum Ca²⁺ refilling during the 10 min exposure to 1.6 mM extracellular Ca²⁺.

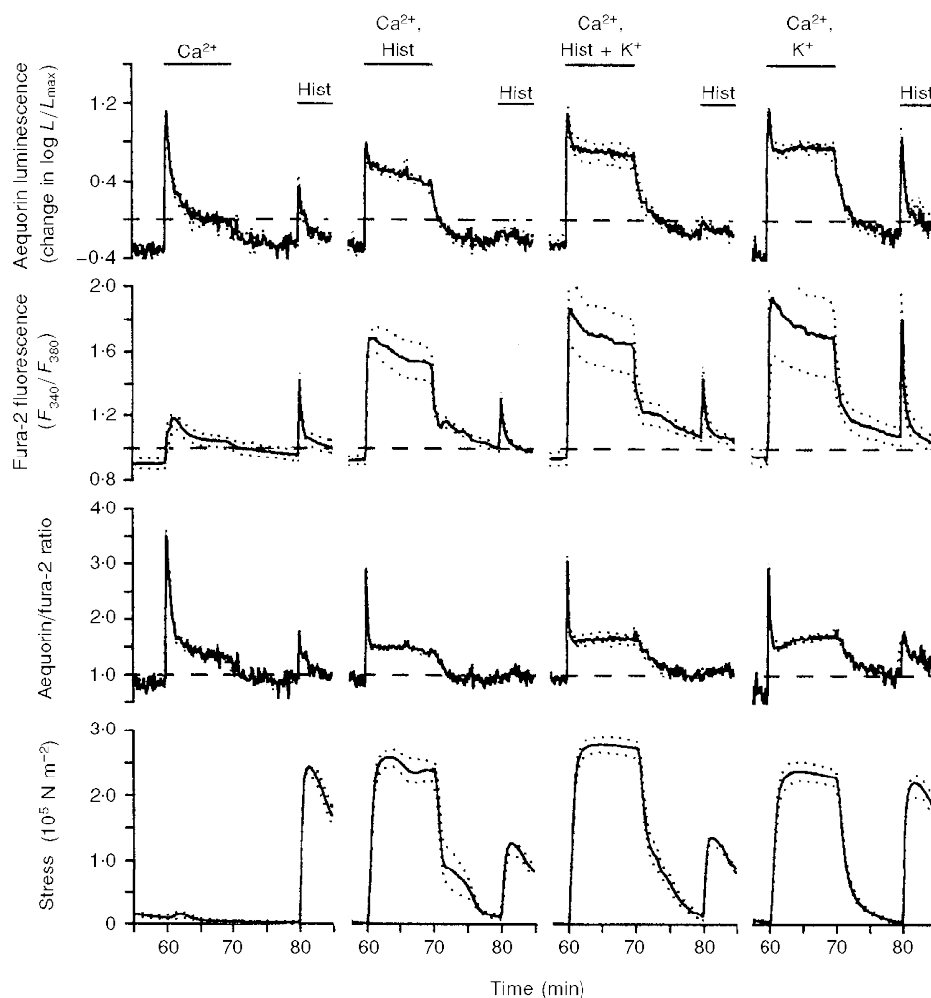


Figure 2. Demonstration that histamine inhibited and high [K⁺]_o enhanced refilling of the sarcoplasmic reticulum with Ca²⁺

Time courses are shown for aequorin-estimated [Ca²⁺]_i, fura-2-estimated [Ca²⁺]_i, the aequorin/fura-2 ratio and active stress. Data are from four sets of swine carotid medial tissues ($n = 4-6$ for each). All tissues had been treated with low extracellular Ca²⁺ and two histamine stimuli as shown in Fig. 1. Each tracing starts at the 57 min time point on Fig. 1, and shows the response to adding back extracellular Ca²⁺ to 1.6 mM at 60 min, then removal of extracellular Ca²⁺ at 70 min, and stimulation with 100 μM histamine (Hist) at 80 min. The first set had Ca²⁺ restored in a normal physiological saline solution (left panel, same data as in Fig. 1). The second set had Ca²⁺ restored in the presence of 100 μM histamine (second from left panel). The third set had Ca²⁺ restored in the presence of 100 μM histamine and 109 mM [K⁺]_o (second from right panel). The fourth set had Ca²⁺ restored in the presence of 109 mM [K⁺]_o (right panel). Data are plotted as in Fig. 1.

The contraction observed in the absence of measurable changes in $[Ca^{2+}]_i$ may result from histamine-induced increases in the $[Ca^{2+}]_i$ sensitivity of myosin light chain phosphorylation at basal $[Ca^{2+}]_i$ levels (Rembold & Murphy, 1988). These data suggest that the modest increases in $[Ca^{2+}]_{pm}$ observed with Ca^{2+} restoration alone (Fig. 3, left panel) were sufficient to partially reload the sarcoplasmic reticulum with Ca^{2+} . This process was inhibited by blocking SERCA with $10 \mu M$ CPA (Fig. 4, left panel).

Restoration of extracellular Ca^{2+} in the presence of CPA, diltiazem and choline⁺ induced a large increase in the aequorin signal with only modest increases in force and the fura-2 signals similar to those observed with diltiazem and

choline⁺ alone. After removal of extracellular Ca^{2+} , histamine stimulation induced a smaller contraction and no change in the aequorin or fura-2 signals, indicating that CPA inhibited Ca^{2+} refilling even with high $[Ca^{2+}]_{pm}$ (the small contraction may represent increases in Ca^{2+} sensitivity). Ca^{2+} restoration in the presence of CPA and choline⁺ induced biphasic increases in both the aequorin and fura-2 signals. The initial increase in the fura-2 signal was larger than that observed with choline⁺ alone. Intriguingly, only the second phase of the increase in fura-2 signal was associated with a contraction. Since the aequorin signal was also high, the initial increase in the fura-2 signal may represent a very high increase in $[Ca^{2+}]_{pm}$ that is detected by both fura-2 and aequorin.

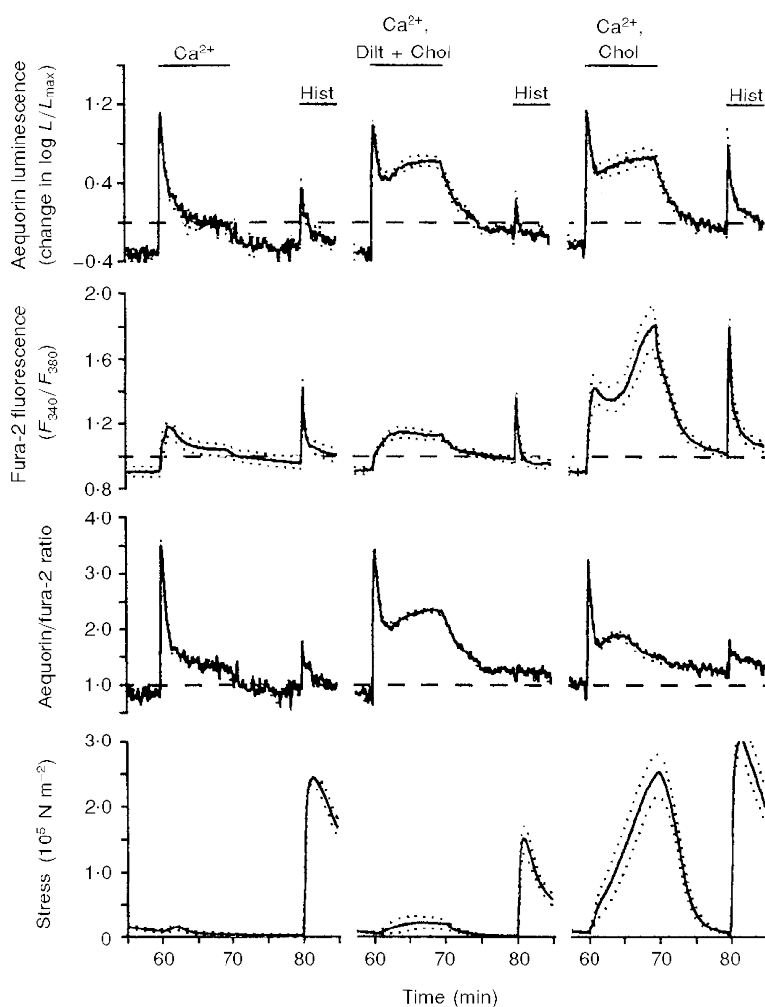


Figure 3. Demonstration that reversal of Na^+ - Ca^{2+} exchange with choline⁺ increased the fura-2 signal and enhanced refilling of the sarcoplasmic reticulum with Ca^{2+} ; both were inhibited by the L-type Ca^{2+} channel blocker diltiazem (Dilt)

Time courses are shown for aequorin-estimated $[Ca^{2+}]_i$, fura-2-estimated $[Ca^{2+}]_i$, the aequorin/fura-2 ratio and active stress. Data are from three sets of swine carotid medial tissues ($n = 4-6$ for each). All tissues had been treated with low extracellular Ca^{2+} and two histamine stimuli as described in Fig. 2. The first set had Ca^{2+} restored in a normal physiological saline solution (left panel, same data as in Fig. 2, left panel). The second set had Ca^{2+} restored in the presence of $10 \mu M$ diltiazem and $140 mM$ choline (substituted isosmotically for Na^+ , centre panel). The third set had Ca^{2+} restored in the presence of $140 mM$ choline (Chol; substituted isosmotically for Na^+ , right panel). Data are plotted as in Fig. 1.

After removal of extracellular Ca^{2+} , repeat histamine stimulation induced a large contraction and small increases in the aequorin and fura-2 signals. These were smaller than observed in the absence of CPA, but suggest that either the CPA did not totally inhibit SERCA or that there are other, CPA insensitive mechanisms responsible for Ca^{2+} uptake into the sarcoplasmic reticulum at high $[\text{Ca}^{2+}]_i$.

Role of the sarcoplasmic reticulum in regulating $[\text{Ca}^{2+}]_i$ homogeneity

Our second hypothesis was that sarcoplasmic reticulum Ca^{2+} handling maintains a $[\text{Ca}^{2+}]_i$ gradient within unstimulated

smooth muscle cells. We evaluated this hypothesis with CPA. In Fig. 4, restoration of extracellular Ca^{2+} in the presence of CPA was associated with a larger increase in force and the fura-2 signal than that observed in the absence of CPA (Fig. 3). These data suggest that Ca^{2+} uptake by the sarcoplasmic reticulum was partially responsible for preventing Ca^{2+} influx from reaching the central cytoplasm where it could induce a contraction (this is the 'buffer barrier' hypothesis). These data suggest a role for the sarcoplasmic reticulum in maintaining a $[\text{Ca}^{2+}]_i$ gradient. We tested this in unstimulated tissues. In the presence of 1.6 mM extracellular Ca^{2+} , addition of 10 μM CPA increased

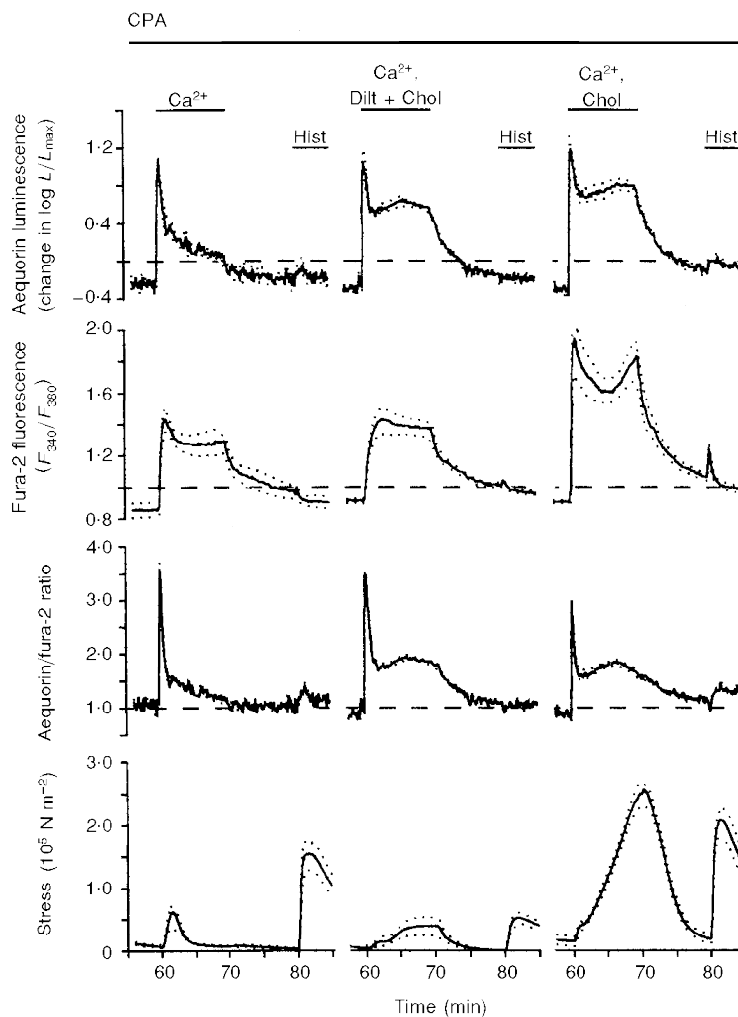


Figure 4. Demonstration that the SERCA inhibitor cyclopiazonic acid (CPA) inhibited choline⁺-induced refilling of the sarcoplasmic reticulum with Ca^{2+} and that CPA increased the fura-2 signal and contractile force during Ca^{2+} restoration

Time courses are shown for aequorin-estimated $[\text{Ca}^{2+}]_i$, fura-2-estimated $[\text{Ca}^{2+}]_i$, the aequorin/fura-2 ratio and active stress. Data are from three sets of swine carotid medial tissues ($n = 4-6$ for each). All tissues had been treated with low extracellular Ca^{2+} and two histamine stimuli as described in Fig. 2. Additionally, 10 μM cyclopiazonic acid (CPA) was added prior to the first histamine stimulation (at 20 min in Fig. 1). This corresponds to 37 min prior to each recording in the current figure. CPA was present throughout the remaining protocol (from 20 to 85 min). The first set had Ca^{2+} restored in a normal physiological saline solution (left panel). The second set had Ca^{2+} restored in the presence of 10 μM diltiazem and 140 mM choline (substituted isosmotically for Na^+ , centre panel). The third set had Ca^{2+} restored in the presence of 140 mM choline (substituted isosmotically for Na^+ , right panel). Data are plotted as in Fig. 1.

the fura-2 signal (Fig. 5), consistent with prior reports (Abe *et al.* 1996). However, CPA did not induce a significant change in the aequorin signal or contractile stress. As a result the aequorin/fura-2 ratio decreased, suggesting that CPA induced a more homogeneous $[Ca^{2+}]_i$ distribution. These data are consistent with the hypothesis that activity of SERCA is at least partially responsible for maintenance of inhomogeneous $[Ca^{2+}]_i$ distribution in unstimulated smooth muscle.

Subsequent addition of 100 μM histamine induced large increases in force and the aequorin and fura-2 signals. The aequorin/fura-2 ratio transiently increased and then decreased back to prior values.

A part of the second hypothesis was that basal levels of Ca^{2+} influx are responsible for elevated $[Ca^{2+}]_{pm}$. We further

evaluated the second hypothesis by studying the response of carotid artery to removal of extracellular Ca^{2+} with or without CPA to inhibit SERCA. The effect of extracellular Ca^{2+} removal with or without cyclopiazonic acid is shown in Fig. 6. Nominal removal of extracellular Ca^{2+} reduced the aequorin signal relatively more than the fura-2 signal (Fig. 6, filled circles; also see Rembold *et al.* 1995). This resulted in a decrease in the aequorin/fura-2 ratio suggesting that removal of extracellular Ca^{2+} induced a more homogeneous $[Ca^{2+}]_i$ distribution. These data are consistent with the second hypothesis that Ca^{2+} influx is responsible for maintaining the Ca^{2+} inhomogeneity in unstimulated smooth muscle. Addition of 10 μM CPA 1 min after nominal removal of extracellular Ca^{2+} induced a significant increase in the fura-2 signal with little change in the aequorin signal. The aequorin signal was similar to the aequorin signal observed with

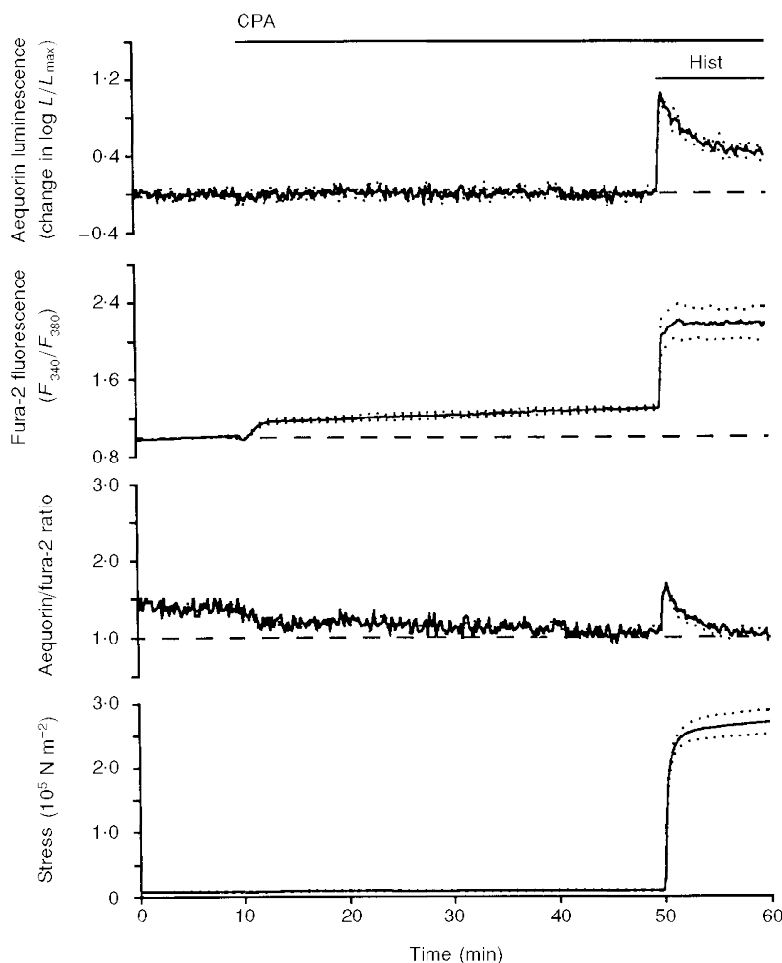


Figure 5. Demonstration that inhibition of SERCA increased the fura-2 signal without altering the aequorin signal; this suggests that SERCA function was responsible for maintaining an inhomogeneous $[Ca^{2+}]_i$ distribution in resting smooth muscle (i.e. maintaining $[Ca^{2+}]_{pm} > [Ca^{2+}]_o$)

Time courses are shown for aequorin-estimated $[Ca^{2+}]_i$, fura-2-estimated $[Ca^{2+}]_i$, the aequorin/fura-2 ratio and active stress. Data are from swine carotid medial tissues ($n = 4-6$). In the presence of 1.6 mM extracellular Ca^{2+} , tissues were first exposed to 10 μM cyclopiazonic acid (CPA) at 10 min and then 100 μM histamine was added at 50 min. Data are plotted as in Fig. 1. The maintenance of maximal contractile stress at peak values despite decreasing aequorin-estimated $[Ca^{2+}]_i$ (cf. 51 vs. 60 min) is a manifestation of the latch phenomenon (Rembold & Murphy, 1988).

removal of extracellular Ca^{2+} without CPA. Addition of CPA transiently decreased the aequorin/fura-2 ratio to values lower than that observed with only removal of extracellular Ca^{2+} . These data are consistent with the hypothesis that the sarcoplasmic reticulum continually 'leaks' Ca^{2+} into the cytosol. Inhibition of SERCA can 'uncover' the Ca^{2+} leak by preventing reuptake of the leaked Ca^{2+} back into the sarcoplasmic reticulum.

Our third hypothesis was that sarcoplasmic reticulum Ca^{2+} handling was important in removal of Ca^{2+} from the centre of the cell upon removal of stimulation (i.e. vectorial Ca^{2+} efflux). The response to histamine stimulation with or without CPA in the absence of extracellular Ca^{2+} is shown in Fig. 7. In both the absence and presence of CPA, 100 μM histamine increased both the aequorin and fura-2 signals

and induced a contraction. There was a transient increase in the aequorin/fura-2 ratio, suggesting the Ca^{2+} release was relatively inhomogeneous. The responses were similar in both the presence and absence of CPA, suggesting that (1) the 10 min CPA treatment had not substantially depleted the sarcoplasmic reticulum of Ca^{2+} and (2) the focal Ca^{2+} release was not significantly modified by CPA-inhibitable SERCA function. Upon washout of histamine, the fura-2 signal from the CPA-treated tissues decreased more slowly than the fura-2 signal from the tissues not treated with CPA. The aequorin signal and contractile stress decreased with a similar time course regardless of the presence of CPA. This result suggests that sarcoplasmic reticulum Ca^{2+} handling is important in reducing $[\text{Ca}^{2+}]_i$ in the central cytoplasm (which is best measured with fura-2). Subplasmalemmal

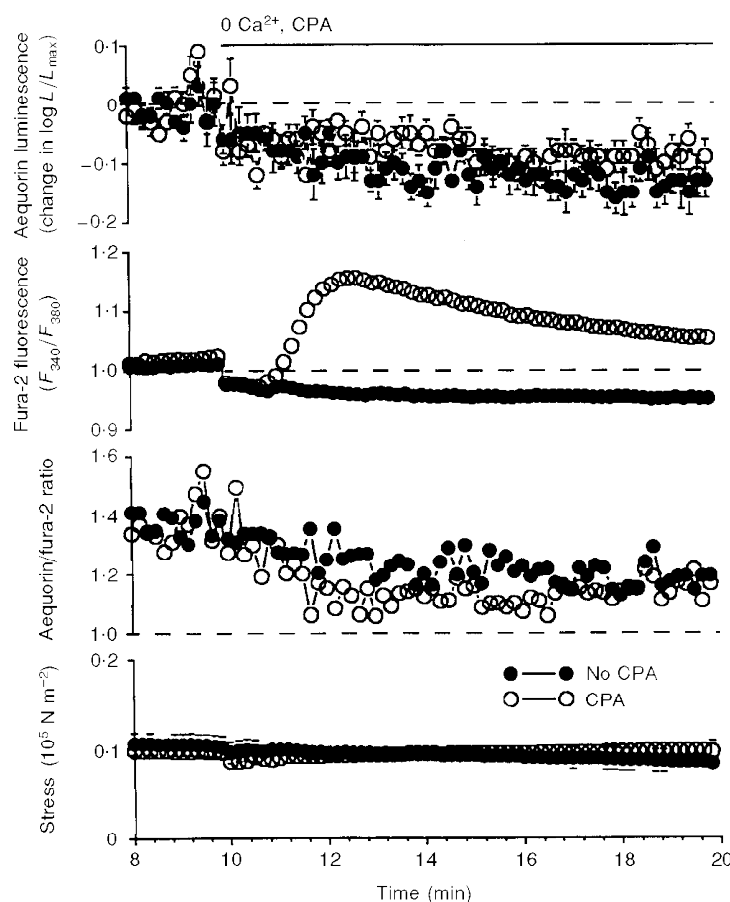


Figure 6. Demonstration that removal of extracellular $[\text{Ca}^{2+}]_o$ reduced the aequorin signal and reduces $[\text{Ca}^{2+}]_i$ inhomogeneity; this suggests that Ca^{2+} influx was responsible for $[\text{Ca}^{2+}]_i$ inhomogeneity in resting smooth muscle.

The data show the response to removal of extracellular Ca^{2+} with and without CPA. Time courses are shown for aequorin-estimated $[\text{Ca}^{2+}]_i$, fura-2-estimated $[\text{Ca}^{2+}]_i$, the aequorin/fura-2 ratio and active stress. Data are means \pm S.E.M. from two sets of swine carotid medial tissues ($n = 4-6$ for each). Extracellular Ca^{2+} was nominally removed at 10 min by changing the bathing solution to a solution with no added Ca^{2+} . At 10.5 min, one set of the tissues was treated with 10 μM CPA (O), and the other set of tissues was not treated with CPA (●). Data were collected at 1 s intervals and averaged over 10 s for plotting. Symbols without error bars reflect errors less than the size of the symbol. Only stress from the fura-2 experiments is shown for clarity (the aequorin stress data were similar).

Ca^{2+} , as measured with aequorin, decreased at a similar rate regardless of the presence of CPA. This is consistent with the hypothesis that Ca^{2+} can be removed from the subplasmalemmal region by the action of plasma membrane Ca^{2+} pumps and Na^+-Ca^{2+} exchange which are not inhibited by CPA.

Our fourth hypothesis was that Ca^{2+} release from the sarcoplasmic reticulum is crucial for the initial phase of agonist-induced contractions. Our goal was to evaluate this hypothesis in the presence of extracellular Ca^{2+} . Therefore, we developed a protocol in which the tissues had varying amounts of Ca^{2+} in the sarcoplasmic reticulum. We accomplished this by removing extracellular Ca^{2+} and then stimulating with varying [histamine] to variably release

Ca^{2+} in the presence of CPA to prevent Ca^{2+} reuptake into the sarcoplasmic reticulum. Controls without CPA were also included. We performed an abbreviated protocol similar to Fig. 1. At 10 min, extracellular Ca^{2+} was nominally removed. At this point, some of the tissues were treated with $10 \mu M$ CPA, which was then present in all subsequent solutions. At 20 min, the tissues were either (1) stimulated with $100 \mu M$ histamine to induce a large release of Ca^{2+} from the intracellular store, (2) stimulated with $3 \mu M$ histamine to induce a more modest Ca^{2+} release, or (3) not stimulated to minimize Ca^{2+} release. At 25 min, the histamine was washed out. At 40 min, extracellular Ca^{2+} was restored to all tissues (see Rembold *et al.* 1995) for a demonstration of this type of experiment). Finally, at 50 min, all tissues were stimulated

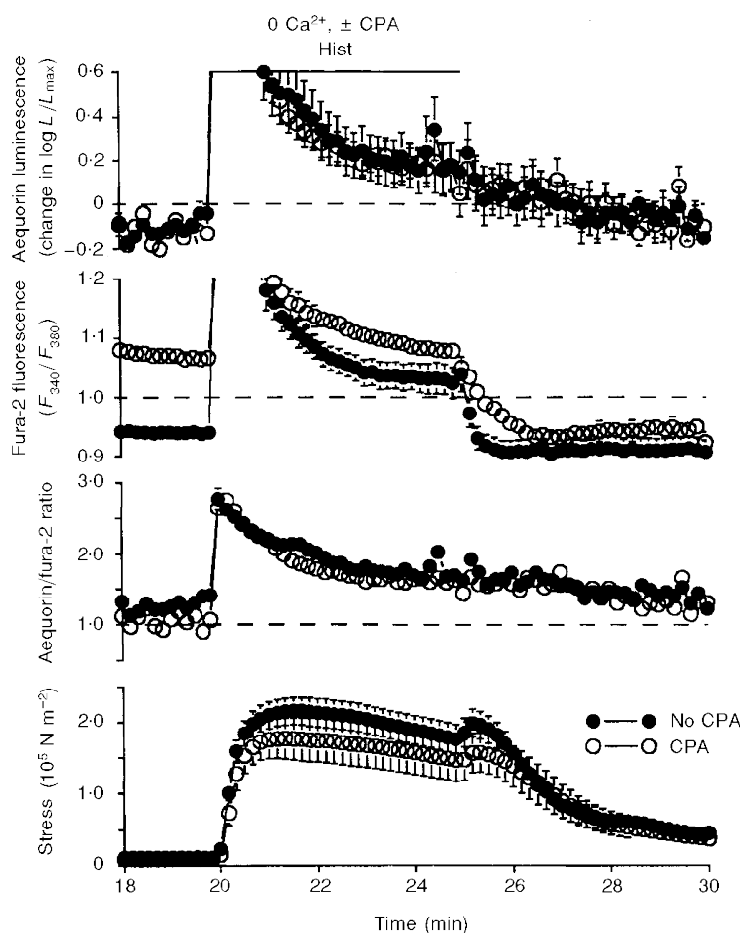


Figure 7. Demonstration that SERCA function was important in vectorial Ca^{2+} efflux; after washout of histamine in the absence of extracellular Ca^{2+} (25 min), cyclopiazonic acid slowed the decline in fura-2-estimated $[Ca^{2+}]_i$.

The data show the response to histamine stimulation with and without CPA in the absence of extracellular Ca^{2+} . Time courses are shown for aequorin-estimated $[Ca^{2+}]_i$, fura-2-estimated $[Ca^{2+}]_i$, the aequorin/fura-2 ratio and active stress. Data are from two sets of swine carotid medial tissues ($n = 4-6$ for each). One set of tissues was treated with $10 \mu M$ CPA (\circ), and the other set of tissues was not treated with CPA (\bullet). The data are a continuation of the experiment shown in Fig. 6. At 10 min, extracellular Ca^{2+} was nominally removed and, at 10.5 min, the first set of tissues was incubated in $10 \mu M$ CPA, which was then present for the remaining protocol. At 20 min, the tissues were stimulated with $100 \mu M$ histamine, and the histamine washed out at 25 min. Data were collected and plotted as in Fig. 6.

with a maximally effective concentration of histamine ($100 \mu\text{M}$). Figure 8 shows the response to the final histamine stimulation depending on prior treatment. There was no significant difference in force or in the aequorin or fura-2 signal during the sustained phase of the contraction (5–10 min after histamine stimulation, data not shown). The only differences were in the first minute after stimulation. The only group showing a delay in force generation was the tissues previously treated with $100 \mu\text{M}$ histamine in the presence of CPA. These data show that depletion of Ca^{2+} from the sarcoplasmic reticulum can transiently attenuate subsequent contraction; however, the effect was relatively small. There were larger effects on the Ca^{2+} signals. The presence of CPA attenuated the histamine-

induced aequorin and fura-2 transients regardless of prior level of histamine stimulation. This is the expected result from CPA-induced inhibition of Ca^{2+} refilling of the sarcoplasmic reticulum. Prior stimulation of CPA-treated tissues with $100 \mu\text{M}$ histamine attenuated the subsequent histamine-induced aequorin and fura-2 transient when compared with the untreated and $3 \mu\text{M}$ histamine-treated tissues. These data suggest that release of Ca^{2+} from the sarcoplasmic reticulum has at least a small role in the initial phase of histamine-induced swine carotid arterial contraction. It appears that other contractile mechanisms can compensate for the loss of sarcoplasmic reticulum Ca^{2+} release with only modest changes in contraction kinetics.

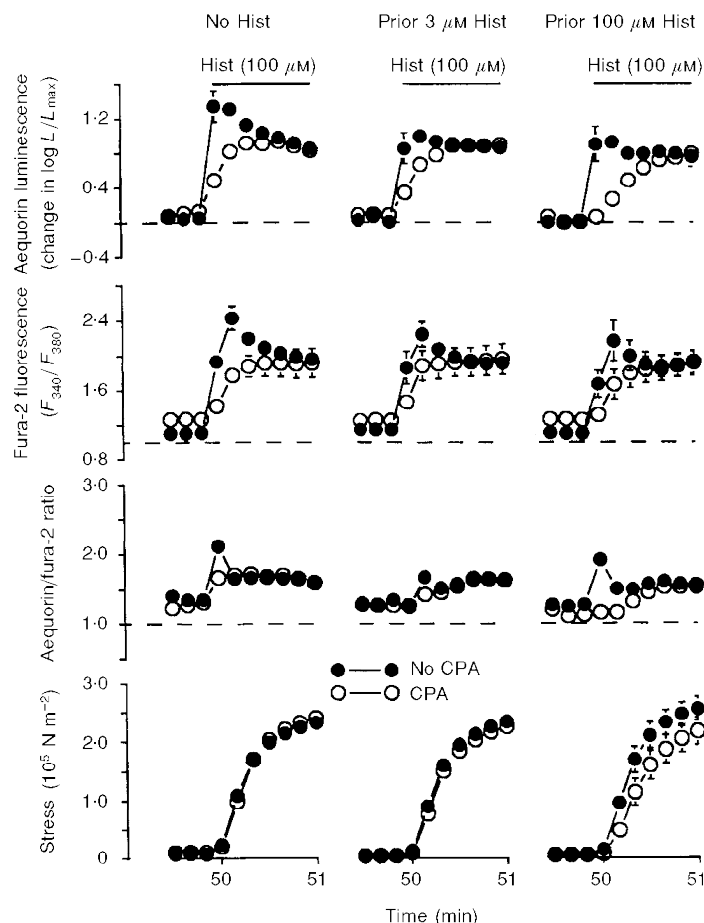


Figure 8. Demonstration that prior depletion of the sarcoplasmic reticulum (with histamine and CPA) modestly attenuated the subsequent histamine-induced Ca^{2+} transient and contraction

Time courses are shown for aequorin-estimated $[\text{Ca}^{2+}]_i$, fura-2-estimated $[\text{Ca}^{2+}]_i$, the aequorin/fura-2 ratio and active stress. Data are from six sets of swine carotid medial tissues ($n = 4-6$ for each). At 10 min, extracellular Ca^{2+} was nominally removed and, at 10.5 min, the three of the six sets of tissues were incubated in $10 \mu\text{M}$ CPA (○) which was then present for the remaining protocol. The other three sets of tissues were not treated with CPA (●). At 20 min, the tissues were then either (1) not stimulated (left panel), (2) stimulated with $3 \mu\text{M}$ histamine (centre panel), or (3) stimulated with $100 \mu\text{M}$ histamine (right panel). The experiments labelled 'prior H100' are a continuation of the experiments shown in Figs 6 and 7. At 25 min, all histamine was washed out. At 40 min, 1.6 mM extracellular $[\text{Ca}^{2+}]$ was restored to all tissues. At 50 min, all tissues were stimulated with $100 \mu\text{M}$ histamine in the presence of 1.6 mM extracellular $[\text{Ca}^{2+}]$; the plot shows 30 s prior to and 60 s after this addition of histamine. Data were collected and plotted as in Fig. 6.

These data also show that prior concerns of histamine-induced aequorin consumption (Rembold & Murphy, 1988; Abe *et al.* 1995) are not a major concern in these experiments. The tissues previously treated with $100 \mu\text{M}$ histamine 25 min before restimulation had aequorin transients (Fig. 8, right panel, filled symbols) only modestly decreased compared with tissues not exposed to histamine stimulation (Fig. 8, left panel, filled symbols).

Finally, we investigated whether inhibition of SERCA altered Ca^{2+} influx. We measured Mn^{2+} -induced quenching of fura-2 fluorescence as a method to estimate Ca^{2+} influx in intact smooth muscle tissues (Chen & Rembold, 1992). Mn^{2+} can traverse L-type Ca^{2+} channels and the Mn^{2+} influx rate through Ca^{2+} channels is proportional to the Ca^{2+} influx rate. Mn^{2+} binding to fura-2 rapidly quenches fura-2 fluorescence and therefore Mn^{2+} binding to fura-2 is not rate limiting. If extracellular Mn^{2+} is added to cells loaded with fura-2, the rate of decline in fura-2 fluorescence (measured at the isobestic wavelength of 360 nm) is proportional to the rate

of Mn^{2+} influx and is an index of the rate of Ca^{2+} influx. We previously found that histamine stimulation or high $[K^+]_o$ depolarization increased Mn^{2+} influx rates and that diltiazem, forskolin and nitroglycerine attenuated histamine-stimulated Mn^{2+} influx (Chen & Rembold, 1992). The protocol was similar to that shown in Fig. 8. At 10 min, extracellular Ca^{2+} was nominally removed. At this point, some of the tissues were treated with $10 \mu\text{M}$ CPA, which was then present in all subsequent solutions. At 20 min, the tissues were either unstimulated or stimulated with $100 \mu\text{M}$ histamine. At 25 min, all histamine was washed out. At 40 min, 0.5 mM $MnCl_2$ was added to the tissues and the fluorescence at 360 nm measured. In the absence of extracellular Ca^{2+} , we found that treatment with CPA and/or prior histamine treatment (to deplete the sarcoplasmic reticulum) had no significant effect on Mn^{2+} influx rates (Fig. 9). As expected, Mn^{2+} influx in the absence of extracellular Ca^{2+} was significantly higher than the Mn^{2+} influx observed in unstimulated controls in the presence of extracellular Ca^{2+} .

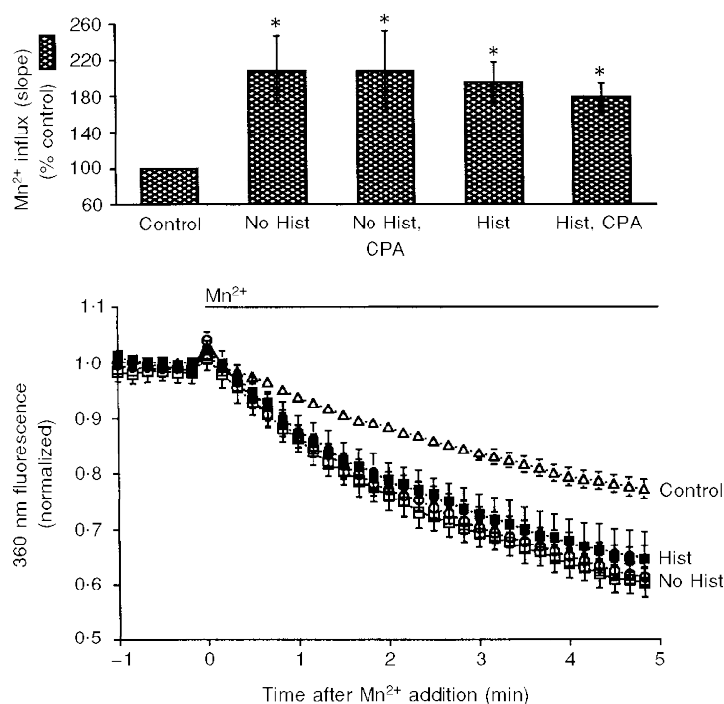


Figure 9. Demonstration that Mn^{2+} influx rates were not changed by depletion of the sarcoplasmic reticulum or the presence of CPA

These data suggest that the Ca^{2+} influx induced by sarcoplasmic reticulum depletion did not enter via Mn^{2+} -permeable channels. In one set of tissues (control), $MnCl_2$ (0.5 mM) was added to tissues without prior manipulation. In the other four sets of tissues, extracellular Ca^{2+} was nominally removed for 10 min. At 10.5 min, the two of the sets were incubated in $10 \mu\text{M}$ CPA which was then present for the remaining protocol. At 20 min, two sets of tissues were stimulated with $100 \mu\text{M}$ histamine for 5 min. At 40 min, $MnCl_2$ (0.5 mM) was added to all four sets of tissues ($n = 4-6$ for each set). The lower panel is the mean (continuous lines) ± 1 s.e.m. (dotted lines) change in 360 nm fluorescence observed after $MnCl_2$ addition to each set of tissues. The time course of change in 360 nm fluorescence was similar in all four restoration studies and all were significantly faster than the control. Lysis refers to the addition of hypotonic 5 mM $MnCl_2$ to determine background fluorescence. Data were collected at 1 s and plotted at 10 s intervals. $*P < 0.05$ vs. control as compared with the Newman-Keuls test.

DISCUSSION

In 1977, Van Breemen observed a dissociation between the rate of $^{45}\text{Ca}^{2+}$ entry and contraction in Ca^{2+} depleted smooth muscle tissues that were activated by restoration of extracellular Ca^{2+} (Van Breemen, 1977). He proposed that addition of extracellular Ca^{2+} increased $[\text{Ca}^{2+}]_i$ focally, i.e. $[\text{Ca}^{2+}]_{\text{pm}}$ increased more than $[\text{Ca}^{2+}]_c$. Van Breemen's hypothesis (termed the 'buffer barrier' hypothesis) requires Ca^{2+} removal by SERCA, the Ca^{2+} pump of the sarcoplasmic reticulum. According to this hypothesis, basal Ca^{2+} influx rates are moderately high in unstimulated smooth muscle cells. Ca^{2+} pumps in the subsarcolemmal sarcoplasmic reticulum and plasma membrane and $\text{Na}^{2+}-\text{Ca}^{2+}$ exchanger in the plasma membrane continually remove Ca^{2+} from the subsarcolemmal space. Ca^{2+} pump activity prevents the increased $[\text{Ca}^{2+}]_{\text{pm}}$ from diffusing to the central cytoplasm where it can increase $[\text{Ca}^{2+}]_c$, activate myosin light chain kinase, and induce a contraction. Ca^{2+} pumps near the plasma membrane are a functional 'barrier' to Ca^{2+} diffusion between the subsarcolemmal space and the bulk cytoplasm. If the sarcoplasmic reticulum is Ca^{2+} depleted, then Ca^{2+} influx into the subsarcolemmal space can refill the sarcoplasmic reticulum without increasing $[\text{Ca}^{2+}]_c$ or inducing a contraction (Van Breemen, 1977; Van Breemen *et al.* 1986). This hypothesis is supported by recent structural studies: electron microscopy reveals that some sarcoplasmic reticulum is near the plasma membrane (Devine *et al.* 1972) and this region contains proteins necessary for Ca^{2+} release and uptake (Villa *et al.* 1993; Moore *et al.* 1993). Such a barrier was predicted by computer models of Ca^{2+} dynamics in smooth muscle cells. These models predicted that intermittently opening Ca^{2+} channel would maintain higher

$[\text{Ca}^{2+}]_{\text{pm}}$ and that Ca^{2+} pump function and passive intracellular Ca^{2+} buffers maintain $[\text{Ca}^{2+}]_c$ at lower levels (Bers & Peskoff, 1991; Kargacin & Fay, 1991).

The goal of this study was to evaluate some of the characteristics of buffer barrier function. The buffer barrier hypothesis predicts several interrelationships between focal changes in $[\text{Ca}^{2+}]_i$ and sarcoplasmic reticulum in intact arterial smooth muscle. We found that sarcoplasmic reticulum function regulates Ca^{2+} homogeneity and that specific types of Ca^{2+} inhomogeneity can determine the degree of Ca^{2+} uptake by the sarcoplasmic reticulum. We evaluated four specific hypotheses. These hypotheses are summarized in Fig. 10.

(1) We hypothesized that increases in $[\text{Ca}^{2+}]_{\text{pm}}$ were primarily responsible for refilling of the sarcoplasmic reticulum with Ca^{2+} . This hypothesis was partially correct. We found that restoration of extracellular Ca^{2+} induced a large increase in the aequorin signal and a smaller increase in the fura-2 signal, suggesting an increase in $[\text{Ca}^{2+}]_{\text{pm}}$ without a large increase in $[\text{Ca}^{2+}]_c$ (Fig. 10, mechanism 1). This increase in $[\text{Ca}^{2+}]_{\text{pm}}$ was associated with partial reloading of the sarcoplasmic reticulum with Ca^{2+} as evidenced by subsequent histamine-induced aequorin and fura-2 transients (Fig. 2, left panel). CPA inhibited reloading, indicating that SERCA function was important for reloading (Fig. 4, left panel). Restoration of extracellular Ca^{2+} in the presence of high $[\text{K}^+]_o$ or choline $^+$ further increased $[\text{Ca}^{2+}]_c$. This increase in $[\text{Ca}^{2+}]_c$ was associated with enhanced reloading of the sarcoplasmic reticulum (right panels in Figs 2, 3 and 10, mechanism 1a). The latter was partly inhibited by CPA (Fig. 4, right panel), suggesting that reloading can also occur

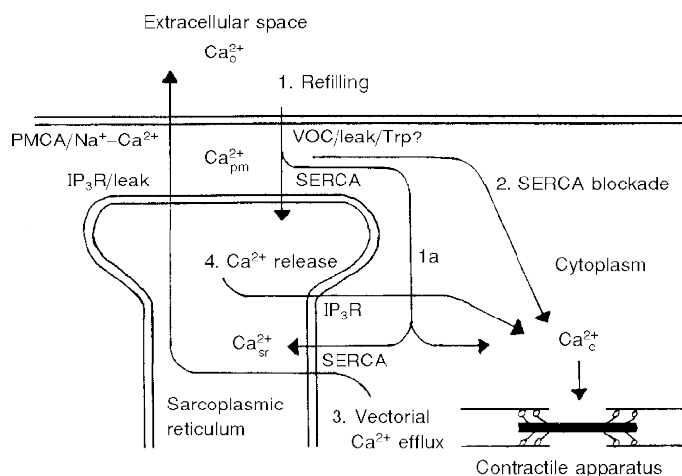


Figure 10. Proposed schema for Ca^{2+} distribution in smooth muscle cells

Shown is a section of a smooth muscle cell showing at the left the extracellular space, at the top the subplasmalemmal space (labelled $[\text{Ca}^{2+}]_{\text{pm}}$), and the sarcoplasmic reticulum. At the bottom right is the cytosol with the contractile apparatus. The proposed movements of Ca^{2+} are shown as arrows numbered 1–4. Abbreviations: PMCA, plasma membrane calcium ATPase (calcium pump); $\text{Na}^+-\text{Ca}^{2+}$, sodium–calcium exchanger; IP_3R , the inositol trisphosphate receptor calcium release channel; Leak, the leak channel; VOC, voltage-operated L-type calcium channel; Trp, the trp channel; SERCA the sarco(endo)plasmic reticulum calcium ATPase (calcium pump); other abbreviations as in text.

via SERCA in the central sarcoplasmic reticulum when $[Ca^{2+}]_c$ is high. Large increases in $[Ca^{2+}]_{pm}$ without large increases in $[Ca^{2+}]_c$, produced by choline⁺ with diltiazem (Fig. 3, centre panel, Fig. 10, mechanism 1), did not enhance reloading of the sarcoplasmic reticulum. These data suggest that sarcoplasmic reticulum Ca^{2+} reloading is bimodal. Transient increases in $[Ca^{2+}]_{pm}$ partially filled the sarcoplasmic reticulum. Larger increases in $[Ca^{2+}]_c$ were required to fully fill the sarcoplasmic reticulum. It is possible that a 'full' sarcoplasmic reticulum is actually 'overloaded.' A full sarcoplasmic reticulum may only be present transiently when Ca^{2+} is being transported vectorially from the central cytoplasm.

(2) We hypothesized that sarcoplasmic reticulum Ca^{2+} handling maintains a $[Ca^{2+}]$ gradient in unstimulated smooth muscle cells. Specifically, we hypothesized that basal levels of Ca^{2+} influx increase subplasmalemmal $[Ca^{2+}]$ ($[Ca^{2+}]_{pm}$) to higher levels than central myoplasmic $[Ca^{2+}]$ ($[Ca^{2+}]_c$) in unstimulated smooth muscle. Blocking SERCA with CPA increased the fura-2 signal without altering the aequorin signal (Figs 5 and 10, mechanism 2). This decreased the aequorin/fura-2 ratio, suggesting a more homogeneous $[Ca^{2+}]_i$ distribution. These data are consistent with a $[Ca^{2+}]_{pm}$ being higher than $[Ca^{2+}]_c$ in resting smooth muscle tissues. Removal of extracellular Ca^{2+} without CPA also reduced the aequorin/fura-2 ratio, suggesting that basal levels of Ca^{2+} influx were responsible for maintaining a higher levels of basal $[Ca^{2+}]_{pm}$ (Fig. 6). These data suggest that one function of the unstimulated sarcoplasmic reticulum is to maintain low $[Ca^{2+}]_c$ even if $[Ca^{2+}]_{pm}$ is higher from high Ca^{2+} influx. These data are consistent with the 'buffer barrier' hypothesis of Van Breemen (Van Breemen, 1977; Van Breemen *et al.* 1986). Similar results were observed when tissues were treated with choline⁺ plus diltiazem to reverse Na^+-Ca^{2+} exchange. In the absence of CPA, there was a large increase in the aequorin signal without a large increase in the fura-2 signal indicating an increase in $[Ca^{2+}]_{pm}$ (Fig. 3). When SERCA was blocked with CPA, the fura-2 signal increased more, indicating that sarcoplasmic reticulum function was necessary to prevent Ca^{2+} in the subplasmalemmal region from diffusing to the central cytoplasm (Figs 4 and 10, mechanism 2). The increased $[Ca^{2+}]_{pm}$ is probably due to reversal of the Na^+-Ca^{2+} exchange as well as to unspecified leak sources (Ashida & Blaustein, 1990). The increased Ca^{2+} in the subsarcolemmal region did not appear to cause increased Ca^{2+} in the bulk cytoplasm, demonstrating that the sarcoplasmic reticulum exerted considerable buffering of incoming Ca^{2+} (Sturek *et al.* 1992).

(3) We hypothesized that the sarcoplasmic reticulum is important in removal of Ca^{2+} from the centre of the cell upon removal of stimulation (Fig. 10, mechanism 3). This has been termed vectorial Ca^{2+} efflux (Stehno-Bittel & Sturek, 1992; Chen & Van Breemen, 1993). Upon washout of histamine, the fura-2 signal from the CPA-treated tissues decreased more slowly than the fura-2 signal from the

tissues not treated with CPA (Fig. 7). This result suggests that sarcoplasmic reticulum Ca^{2+} handling is important in reducing $[Ca^{2+}]$ in the central cytoplasm, which is best measured with fura-2. Vectorial Ca^{2+} efflux may be important in reducing $[Ca^{2+}]_c$ after high levels of stimulation such as high $[K^+]_o$ (Fig. 2). Subplasmalemmal Ca^{2+} , as measured with aequorin, decreased at a similar rate regardless of the presence of CPA. This is consistent with the hypothesis that Ca^{2+} is removed from the subplasmalemmal region by the action of plasma membrane Ca^{2+} pumps and Na^+-Ca^{2+} exchange, which are not inhibited by CPA.

(4) We hypothesized that Ca^{2+} release from the sarcoplasmic reticulum is crucial for the initial phase of agonist-induced contractions (Fig. 10, mechanism 4). Sarcoplasmic reticulum Ca^{2+} depletion by prior treatment with 100 μM histamine and CPA transiently attenuated the response to subsequent histamine stimulation (Fig. 8). Histamine-induced aequorin and fura-2 transients were substantially attenuated. The effect on contraction was smaller: a delay in contraction of approximately 10 s. These data suggest that release of Ca^{2+} from the sarcoplasmic reticulum has at least a small role in the initial phase of histamine-induced swine carotid arterial contraction. It appears that other contractile mechanisms can compensate for the loss of sarcoplasmic reticulum Ca^{2+} release with only modest changes in contraction kinetics.

These data suggest that the sarcoplasmic reticulum has an important role in regulating $[Ca^{2+}]$ in at least two cytoplasmic compartments of smooth muscle cells. In unstimulated smooth muscle, Ca^{2+} pumping by SERCA maintains lower $[Ca^{2+}]_c$ despite basal levels of Ca^{2+} influx which increase $[Ca^{2+}]_{pm}$. After stimulation, Ca^{2+} pumping by SERCA is important for reductions in $[Ca^{2+}]_c$. Agonist-induced Ca^{2+} release can induce contraction; however, if this mechanism is inhibited, there are other contractile mechanisms that can initiate and maintain contraction without a large effect on contractile kinetics. These data are consistent with Van Breemen's 'buffer barrier' hypothesis (Van Breemen, 1977; Van Breemen *et al.* 1986).

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