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The superficial buffer barrier in venous smooth muscle: sarcoplasmic reticulum refilling and unloading

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1 The interaction of Ca^{2+} transport in the plasmalemma and the sarcoplasmic reticulum (SR) was investigated in smooth muscle of the rabbit inferior vena cava. We tested the possibility of direct refilling of the SR with extracellular Ca^{2+} and of the existence of a vectorial Ca^{2+} extrusion pathway from the SR lumen to the extracellular space suggested by earlier results.

2 After depletion with caffeine the SR was loaded with Ca^{2+} to increasing levels by incubation in a high potassium 1.5 mM Ca^{2+} solution and a 10 mM Ca^{2+} zero Na^+ solution, respectively. Thapsigargin, 2 μM, (a specific SR Ca^{2+}-ATPase blocker) completely blocked refilling of the SR in either of the above solutions, indicating that the SR Ca^{2+}-ATPase is essential for this process.

3 Three different agents, caffeine, ryanodine and thapsigargin, which inhibit Ca^{2+} accumulation by the SR, increased the steady state intracellular Ca^{2+} concentration in the rabbit inferior vena cava.

4 Measurements of Mn^{2+} induced quenching of the intracellular fura-2 signal during pharmacological manipulation of the SR content showed that these three agents did not stimulate divalent cation entry.

5 On the other hand, stimulation with noradrenaline caused a marked increase in Mn^{2+} influx, which was blocked by 2 mM Ni^{2+}. Mn^{2+} entry stimulated by high K^+ solution was blocked by 1 μM diltiazem.

6 We conclude that the SR refilling has to be mediated by the SR Ca^{2+}-ATPase. Inhibition of Ca^{2+} accumulation by the SR causes an increase in the steady state intracellular Ca^{2+} concentration. This observation cannot be explained by an increase in Ca^{2+} influx into the smooth muscle cells of the rabbit inferior vena cava. Alternatively these results suggest the existence of a continuous vectorial release of Ca^{2+} from the SR lumen to the extracellular space.

Keywords: Smooth muscle: plasmalemma; sarcoplasmic reticulum (SR); intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$; fura-2; Mn^{2+} quenching; superficial buffer barrier (SBB)

Introduction

The sarcoplasmic reticulum (SR) of vascular smooth muscle functions both as a source and a sink of activator Ca^{2+}. The 100 kD Ca^{2+}-ATPase (Eggermont *et al.*, 1988) pumps Ca^{2+} from the cytoplasm into the SR lumen to promote relaxation and to load it for subsequent release during activation. Ca^{2+} release is mediated by inositol (1,4,5) trisphosphate (Ins-(1,4,5)P_3-sensitive Ca^{2+} channels and others sensitive to both Ca^{2+} and ATP (Saida, 1982; Saida & van Breemen, 1984; Suematsu *et al.*, 1984; Meissner *et al.*, 1986). In addition to these well established roles, the peripherally located SR may also function as a 'buffer barrier' to Ca^{2+} entry into the deeper myoplasm.

In essence the SR 'buffer barrier' hypothesis states that: (1) Ca^{2+} which enters the cell across the plasmalemma is in part pumped into the SR before it can activate contraction; (2) the Ca^{2+}-ATPase of the SR contributes to Ca^{2+} extrusion from the cells due to vectorial release of SR Ca^{2+} towards the inner surface of the plasmalemma, from where it is extruded by the Na^+/Ca^{2+} exchanger and the plasmalemmal Ca^{2+}-ATPase; and (3) a Ca^{2+} gradient in the peripheral cytoplasm is created by these processes (Figure 8). Experimental support for the 'buffer barrier' hypothesis is derived from a number of studies made over the last 15 years. The initial finding that the magnitude of contraction was more closely related to the rate than the magnitude of net Ca^{2+} entry into aortic smooth muscle suggested that the superficial SR sequestered Ca^{2+} before it could reach the myofilaments to activate them (van Breemen, 1977). In a more recent paper (Chen *et al.*, 1992a), we reported that depletion of the SR induced a delay between the rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) due to stimulation of Ca^{2+} influx and the simultaneously measured force development. This result could be explained if it was assumed that the depletion of the SR enhances its effectiveness in removing Ca^{2+} which enters the cells. This explanation was supported by the observation that caffeine, which prevents the SR buffering action by maintaining the Ca^{2+} channels on the SR in an open state, abolished the above delay. Thus it appears that the unloaded SR can effectively buffer Ca^{2+} which enters the cells. Maintenance of such a buffer barrier function would require the transfer of Ca^{2+} from the SR lumen to the extracellular space (ECS). Recent evidence derived from electrophysiological studies and $[Ca^{2+}]_i$ measurements lends support to the concept of vectorial unloading of the SR (Stehno-Bittel & Sturek, 1992; Chen *et al.*, 1992a). This aspect of Ca^{2+} homeostasis will be explored further in the present paper.

Functional connections between the plasmalemma and the SR have been proposed before; however, mostly in order to explain SR refilling from the ECS (Brading *et al.*, 1980; Casteels & Droogmans, 1981; Putney, 1986; Chen *et al.*, 1992b). In fact Casteels & Droogmans (1981) and the groups of Putney (Putney, 1986) and Daniel (Bourreau *et al.*, 1992) have postulated a direct pathway between the ECS and SR or ER lumen which can bypass the SR Ca^{2+}-ATPase. Alternatively the SR may be refilled by virtue of the SR Ca^{2+} pump removing Ca^{2+} from a restricted cytoplasmic space adjacent to the plasmalemma consistent with the buffer barrier hypothesis. In this study, we have employed the SR Ca^{2+}-ATPase inhibitor, thapsigargin, to distinguish between these two possible mechanisms in smooth muscle of the rabbit inferior vena cava.

A preliminary account of these results was presented at the International Symposium on Smooth Muscle, held January 29–February 1, 1992 in Fukuoka, Japan.

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Methods

Tissue preparation

Male New Zealand white rabbits weighing 1.5–2.5 kg were killed by CO_2 asphyxiation followed by exsanguination from the carotid arteries. The inferior vena cava was rapidly excised and placed in warmed (37°C), oxygenated (100%) physiological saline solution (PSS) (pH 7.4). The vena cava was then cleaned of connective tissue and fat and opened longitudinally. The endothelium was removed by gently rubbing the intimal surface with a piece of wet filter paper. Acetylcholine failed to relax the inferior vena cava in which tension development had been stimulated with noradrenaline (NA) (data not shown). The inferior vena cava was subsequently cut into a rectangular strip approximately 7 mm in width and 15 mm in length and tied to a stainless steel tissue holder.

$[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ in the inferior vena cava was measured as described by Nishimura *et al.* (1989). Briefly, the mounted preparation was placed in a 3.5 ml polystyrene cuvette containing PSS, and positioned in the temperature-controlled (37°C) chamber of the spectrofluorimeter (Spex Fluorolog, Spex Industries, New Jersey, U.S.A.) to measure the autofluorescence. The luminal side of the tissue was illuminated with alternating wavelengths of 340 and 380 nm controlled by an electronic chopper, and 505 nm emitted light from the strip was collected in the front face mode via a photomultiplier. Excitation at either 340 or 380 nm was synchronized with emission by software designed by Spex Industries. The mounted muscle strip was then loaded with fura-2 by incubation in the dark in a solution containing 5 μM fura-2/AM, 0.5% cremophor, and 3 mg l^{-1} bovine serum albumin for 2–3 h at room temperature (<25°C). The tissue was then returned to the chamber and washed repeatedly for 30 min with PSS (37°C) to remove excess external fura-2/AM.

After the autofluorescences at 340 and 380 nm were subtracted, the ratio of fluorescence due to excitation at 340 nm (F340) to that at 380 nm (F380) was calculated.

When we use the equation of Grynkiewicz *et al.* (1985) to calculate $[Ca^{2+}]_i$, the resting $[Ca^{2+}]_i$ of the rabbit inferior vena cava smooth muscle cells in normal PSS was about 80 nM which is compatible with the values measured by others.

Grynkiewicz's equation:

$$[Ca^{2+}]_i = (K_d)(b)[R - R_{min}]/[R_{max} - R]$$

In the equation, K_d is the dissocation constant of Ca^{2+}-fura-2 complex; R is the above-mentioned ratio of fluorescence due to excitation at 340 nm to that at 380 nm; R_{min} and R_{max} are the ratios measured by the addition of 10 μM of the Ca^{2+} ionophore ionomycin to Ca^{2+}-free (10 mM EGTA) solution and Ca^{2+}-replete (2 mM $CaCl_2$) solution respectively; b is the ratio of the 380 nm signals in Ca^{2+}-free and Ca^{2+}-replete solution. There are several reasons for use of the ratio F340/F380 as a relative measurement of $[Ca^{2+}]_i$ instead of calculating $[Ca^{2+}]_i$ with the above equation: (1) It is very difficult to determine the K_d value in the cytoplasm. Simply applying the K_d measured in the salt solution to the above equation will not reflect the real $[Ca^{2+}]_i$. (2) The intensity of fura-2 fluorescence in the cell-free solution might be different from that in the cytoplasm. (3) Employment of ionomycin does not seem to permeabilize completely the smooth muscle cells to Ca^{2+} in our preparation and therefore cannot give satisfactory R_{max} and R_{min}.

At 360 nm wavelength, the fluorescence signal was not influenced by Ca^{2+} concentration changes. Mn^{2+} quenching of fura-2 fluorescence at this wavelength was therefore used to measure the rate of Mn^{2+} influx by measuring the slope of Mn^{2+} quenching trace.

Solutions and chemicals

Normal PSS (N-PSS) contains (in mM): NaCl 140, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1, glucose 10, HEPES 5, pH 7.4. 0 Ca^{2+} PSS was almost the same as normal PSS except that no $CaCl_2$ was added to the solution. In Ca^{2+}-free PSS, 0.1 mM EGTA replaced 1.5 mM $CaCl_2$. For 80 K^+-PSS, 75 mM NaCl was replaced with equimolar KCl to make the final KCl concentration equal to 80 mM. In 0 Ca^{2+}/80 K^+-PSS, EGTA was added to and $CaCl_2$ was omitted from the 80 K^+ PSS. The 0 Na^+/10 Ca^{2+} PSS was prepared by substituting LiCl for NaCl and raising the calcium concentration to 10 mM.

Drugs were obtained from the following sources: caffeine (Sigma), thapsigargin (LC Service Corporation), monensin (Calbiochem), ouabain (Sigma), ryanodine (a gift from Dr M.T. Nelson) and noradrenaline (NA) (Sigma).

Results

Thapsigargin prevents the SR refilling with Ca^{2+}

Application of caffeine or physiological agonists, e.g. NA and histamine, in the absence of Ca^{2+} has proved to be an effective tool to deplete SR and to estimate SR Ca^{2+} content in smooth muscle cells (van Breemen, 1976). In the experiments displayed in Figure 1a and 1b, the SR was first depleted by 25 mM caffeine in 0 Ca^{2+}-PSS. The tissue was then exposed to either high K^+ solution or a high Ca^{2+}, Na^+-free medium. Large Ca^{2+} influxes were induced under these conditions through voltage-gated Ca^{2+} channels (VGC) and the Na^+/Ca^{2+} exchanger. In the absence of thapsigargin, the SR was refilled with Ca^{2+} in both cases as indicated by the second caffeine-induced Ca^{2+} signals, which were larger than the controls (upper panels of Figure 1a and b). Thapsigargin, a specific SR Ca^{2+}-ATPase blocker, prevented the SR refilling through both pathways, as indicated by the failure of caffeine to induce a second $[Ca^{2+}]_i$ peak regardless of whether Ca^{2+} influx was mediated by VGC or Na^+/Ca^{2+} exchanger (lower panels of Figure 1a and b).

These results indicate that in the rabbit inferior vena cava, the SR has to be refilled via the SR Ca^{2+}-ATPase and do not support the existence of a direct connection between the SR lumen and the ECS.

Selective inhibition of Ca^{2+} accumulation by the SR increases steady state $[Ca^{2+}]_i$

Three different agents, caffeine, ryanodine and thapsigargin, all enhanced the steady state $[Ca^{2+}]_i$ (Figure 2a,b,c). These three agents have in common that they inhibit Ca^{2+} accumulation by the SR. Caffeine and ryanodine open Ca^{2+} channels in the SR (Rousseau & Meissner, 1987; 1989) and cause fast and slow release of Ca^{2+} from the SR, respectively (van Breemen, 1976; Hwang & van Breemen, 1987). Thapsigargin disrupts the balance between Ca^{2+} leak and Ca^{2+} uptake through the SR membrane at rest, by blocking the 100 kD Ca^{2+}-ATPase and thus also empties the SR. The three agents were used in separate experiments to avoid possible misleading conclusions based on side effects of any particular agent. The results thus lead to the conclusion that selective inhibition of Ca^{2+} accumulation by the SR causes an increase in the steady state $[Ca^{2+}]_i$. Each experiment described above was repeated four or five times and the means of the relative steady state Ca^{2+} concentrations are depicted in Figure 2d.

The elevation in steady state $[Ca^{2+}]_i$ is caused either by an increase in Ca^{2+} influx or a decrease in Ca^{2+} efflux. Putney (1990) proposed that SR depletion causes an increase in plasmalemmal Ca^{2+} permeability by an as yet unspecified mechanism. Since this hypothesis enjoys broad support, its possible application to venous smooth muscle was investigated by measurement of divalent cation influx.

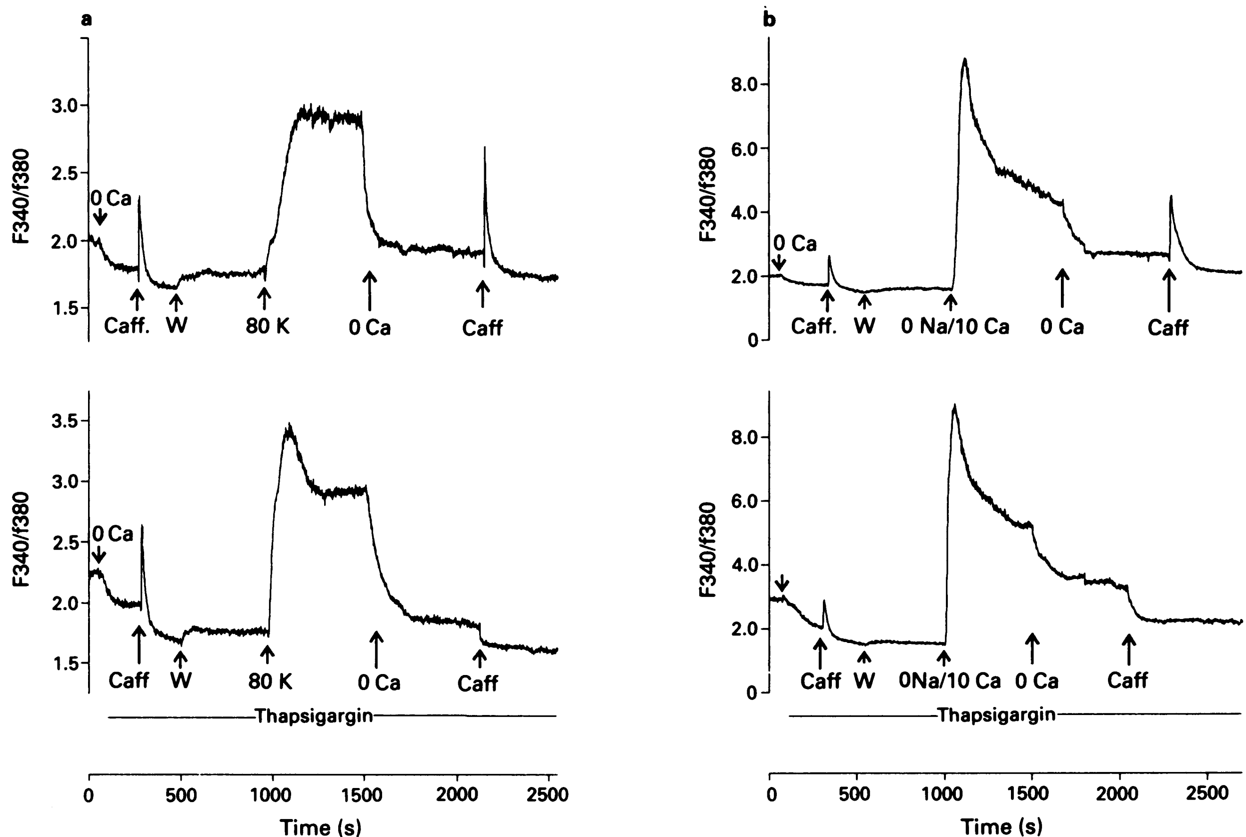


Figure 1 (a) Thapsigargin blocks sarcoplasmic reticulum (SR) refilling by Ca^{2+} influx through VGC (upper panel). Caffeine (Caff, 25 mM) was added for 3 min in the absence of Ca^{2+} in order to deplete the SR. A $[Ca^{2+}]_i$ peak was induced during this process. After 80 K^+ solution was applied, the $[Ca^{2+}]_i$ signal increased to an elevated steady state level. 80 K^+ solution was then replaced by 0 Ca^{2+}-PSS causing a decline in F340/F380. An even higher $[Ca^{2+}]_i$ peak was induced by the second application of caffeine, which indicated that the SR was loaded with Ca^{2+} during incubation in 80 K^+-PSS (lower panel). The experimental protocol was the same as above, except that thapsigargin (2 μM) was included in all the solutions used. Caffeine failed to induce a second peak in the presence of thapsigargin indicating that refilling was prevented. (b) Thapsigargin blocks the SR refilling by Ca^{2+} influx through the Na^+/Ca^{2+} exchanger (upper panel). The experimental protocol is similar to that in (a), but 0 Na^+, 10 mM Ca^{2+} solution was used instead of 80 K^+ solution during the refilling period. Ouabain (100 μM) and monensin (120 μM) were included in the solutions throughout the experiment in order to intensify the activity of Na^+/Ca^{2+} exchanger by loading the cell with Na^+. A caffeine-induced $[Ca^{2+}]_i$ signal peak was seen upon SR depletion. The large second peak indicates a high degree of SR loading upon Na^+ gradient reversal in the presence of 10 mM Ca^{2+}. (lower panel) Including 2 μM thapsigargin in an otherwise identical protocol blocked reloading of the SR as witnessed by the absence of a second caffeine-induced $[Ca^{2+}]_i$ signal peak. Thapsigargin completely blocked the second caffeine induced Ca^{2+} transient in 4 out of 4 experiments.

Mn^{2+} as a probe for Ca^{2+} influx in the rabbit inferior vena cava

Mn^{2+} ions have previously been shown to be a good substitute for Ca^{2+} ions in defining Ca^{2+} influx pathways (Missiaen *et al.*, 1990). They pass through almost all the Ca^{2+} channels in the plasmalemma, but cannot be taken into the SR by the SR Ca^{2+}-ATPase (Gomes da Costa & Madeira, 1986). In the rabbit inferior vena cava, Mn^{2+} entered the smooth muscle cells and quenched the fura-2 fluorescence in a linear manner, at least until 50% of the fluorescence was quenched (Figure 3). Thus, the slope of the first part of the quenching trace can be regarded as a measure of the rate of Mn^{2+} entry into the cells (Figure 3 legend).

Besides the leak pathway, Mn^{2+} also enters the plasmalemma through voltage-gated Ca^{2+} channels (VGC). As shown in Figure 4, high K^+ solution opened the VGC by depolarizing the membrane potential and caused an increase in the rate of Mn^{2+} influx. This effect was completely blocked by 1 μM diltiazem, a known VGC blocker (Figure 4).

NA is believed to stimulate opening of the receptor-operated channels (ROC) and facilitate the opening of VGC (reviewed by van Breemen & Saida, 1989). It also increased the rate of Mn^{2+} entry into the smooth muscle cells (Figure 5a). The VGC blocker, diltiazem, only partially inhibited the effect of NA (Figure 5b), but 2 mM Ni^{2+}, a blocker of both VGC and ROC, totally blocked the effect of NA on increasing the Mn^{2+} influx (Figure 5c).

The results above indicate that Mn^{2+} can enter the smooth muscle cells through the non-regulated leak pathway, VGC and ROC which are the three most important Ca^{2+} entry pathways in the smooth muscle plasmalemma.

Caffeine, ryanodine and thapsigargin do not increase the plasmalemmal permeability of divalent cations

As indicated above, caffeine, ryanodine and thapsigargin all enhanced the steady state $[Ca^{2+}]_i$. However none of these agents seemed to do so by increasing Ca^{2+} entry through the plasmalemma, as reflected by their failure to increase the rate of Mn^{2+} influx (Figure 6a,b,c). Therefore, we conclude that SR depletion by itself is not sufficient to cause an increase in plasmalemmal divalent cation permeability in the smooth muscle of the rabbit inferior vena cava.

We repeated each Mn^{2+} experiment 4 to 5 times and the average results are depicted in Figure 7.

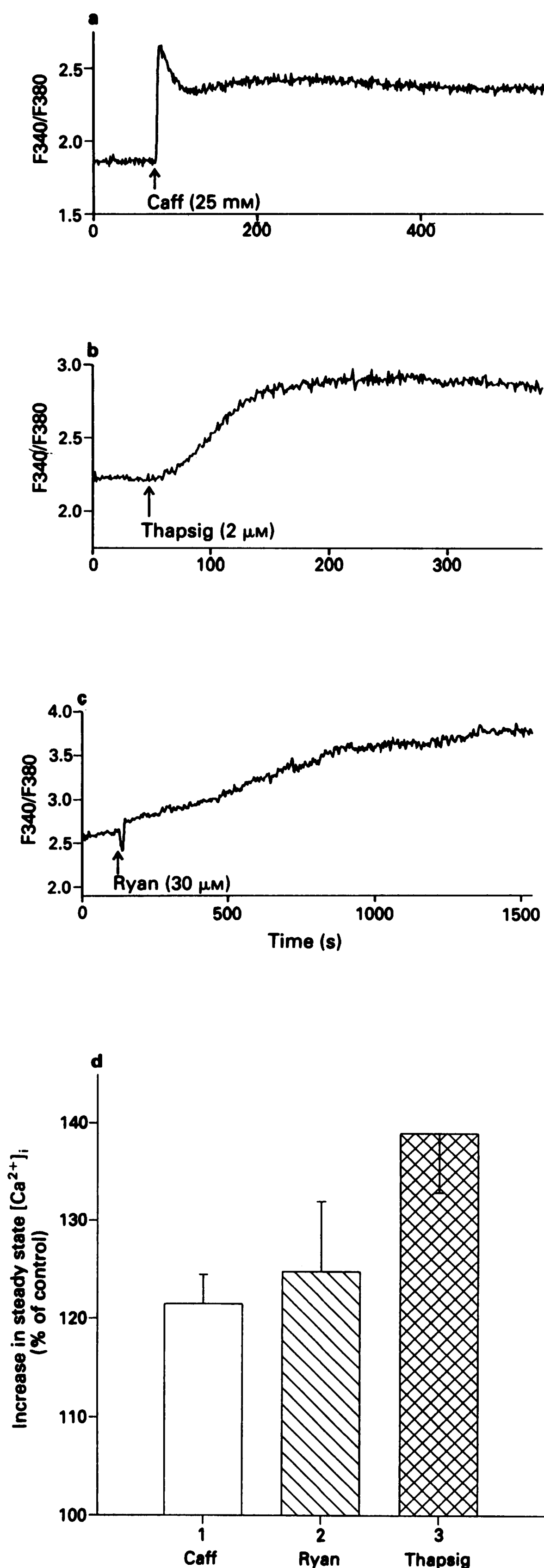


Figure 2 Three different agents which inhibit Ca^{2+} accumulation by sarcoplasmic reticulum (SR) shifted the steady state $[Ca^{2+}]_i$ seen in normal PSS to a higher value: (a) 25 mM caffeine (Caff); (b) 30 μM ryanodine (Ryan); (c) 2 μM thapsigargin (Thapsig). (d) A summary of the effects of above three agents on increasing the steady state $[Ca^{2+}]_i$. Values represent the percentage change in $[Ca^{2+}]_i$ compared with the respective control group. Each column represents the average of 4 experiments ± s.e. (1) Caff, 25 mM caffeine; (2) Ryan, 30 μM ryanodine; (3) Thapsig, 2 μM thapsigargin.

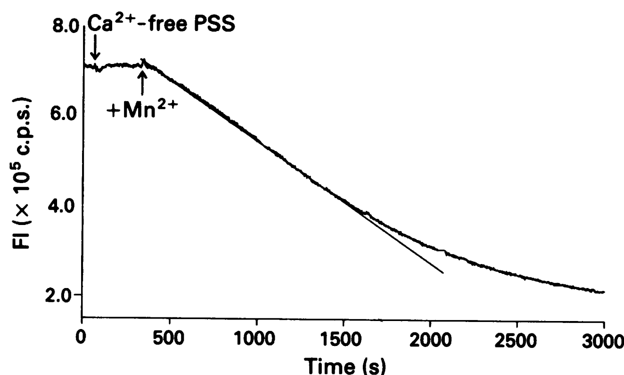


Figure 3 Mn^{2+} quenching of fura-2 fluorescence at excitation wavelength 360 nm. The incubating solution PSS was first replaced with Ca^{2+}-free PSS. The fluorescence intensity decayed immediately upon additon of 100 μM Mn^{2+}. After less than 1 min, the quenching trace appeared to be a straight line and finally levelled off when the fluorescence decayed to 50% of its original level. The slope of the straight line portion of the fura-2 quenching trace was assumed to be proportional to the rate of Mn^{2+} entry. All the subsequent Mn^{2+} quenching recordings were terminated before the fluorescence intensities decayed to 50% of their original values. (The ordinate scale indicates fura-2 fluorescence intensity excitation at 360 nm).

Discussion

After the SR Ca^{2+} has been depleted by exposure to a full agonist or caffeine in Ca^{2+}-free solution, it can be refilled by Ca^{2+} entering the cells from the ECS (Aaronson & van Breemen, 1981). Our data demonstrated that the SR Ca^{2+}-ATPase is essential for this process, since a blocker of the SR Ca^{2+}-ATPase, thapsigargin, completely prevented the SR refilling even if a large Ca^{2+} influx was induced through either excitable Ca^{2+} channels or the Na^+/Ca^{2+} exchanger (Figure 1a and b). These results suggest that during refilling of the SR, Ca^{2+} first enters the cytoplasm and is then actively transported into the SR lumen. On the other hand Casteels & Droogmans (1981) argued that since relatively large quantities of Ca^{2+} could be transferred from the ECS to the SR without contraction, there might be a direct Ca^{2+} transport pathway between these Ca^{2+} compartments. Their group has recently provided evidence for the participation of such a direct pathway in the refilling of the SR in A7r2 cells (a cell line derived from embryonic rat aorta) (Missiaen *et al.*,

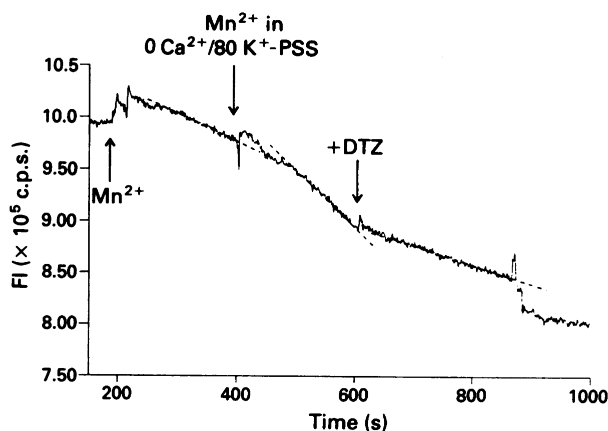


Figure 4 Effect of high K^+ solution on the rate of Mn^{2+} entry: the inferior vena cava was sequentially exposed to PSS, Ca^{2+}-free PSS, 100 μM Mn^{2+} added to Ca^{2+}-free PSS, Mn^{2+} added to 0 Ca^{2+}, 80 mM K^+-PSS and the same with the addition of 1 μM diltiazem (DTZ). High K^+ solution increased the rate of Mn^{2+} entry into the cells and this effect was completely blocked by 1 μM diltiazem. (The ordinate scale indicates fura-2 fluorescence intensity excitation at 360 nm).

1990). After depleting the SR of Ca^{2+} in the A7r5 cells, they incubated them in a Ca^{2+}-free, $^{54}Mn^{2+}$-containing medium for several minutes. Following subsequent saponin permeabilization, $^{54}Mn^{2+}$ efflux from the intracellular stores could be detected. Since Mn^{2+} permeates the plasmalemma but cannot be taken up by the SR Ca-ATPase (Gomes da Costa & Madeira, 1986), they concluded that the SR Ca^{2+} pump was not essential for SR refilling and that there must be a direct pathway for Ca^{2+} between the SR lumen and the ECS (Missiaen *et al.*,

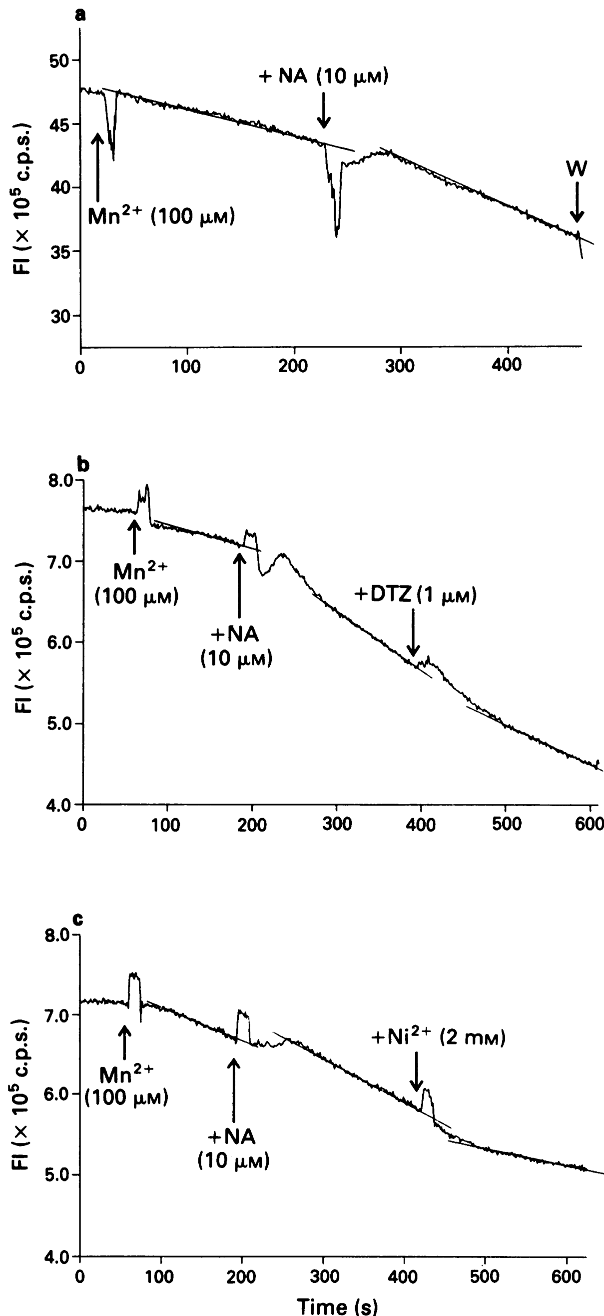


Figure 5 Effect of noradrenaline (NA) on the rate of Mn^{2+} entry. (a) After incubating the inferior vena cava in PSS, 25 mM caffeine in Ca^{2+}-free PSS was first applied to deplete the sarcoplasmic reticulum (SR) of Ca^{2+} in order to reduce the tissue mobility. The caffeine was then washed away with Ca^{2+}-free PSS. Mn^{2+} and NA were added consecutively at the indicated times. (b) The protocol was the same as above with the addition of one more step when 1 µM diltiazem (DTZ) was added in addition to Mn^{2+} and NA. Diltiazem partially inhibits the effect of NA on increasing the rate of Mn^{2+} entry. (c) The protocol was the same as that in (b) with the exception that 2 mM Ni^{+} instead of 1 µM diltiazem completely blocked the effect of NA on the rate of Mn^{2+} entry. (Ordinates indicate fura-2 fluorescence intensities excitation at 360 nm).

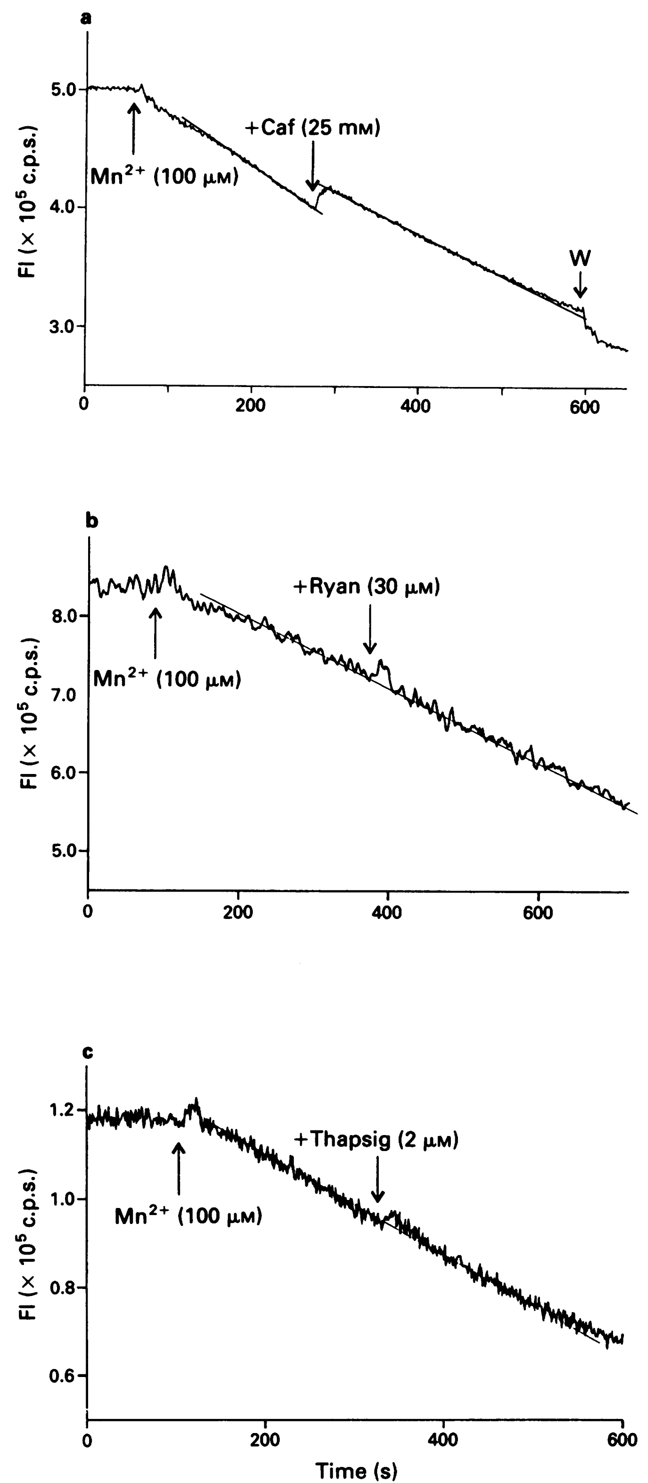


Figure 6 The effects of agents which inhibit Ca^{2+} accumulation by the sarcoplasmic reticulum (SR) on the rates of Mn^{2+} quenching of fura-2 fluorescence. (a) The rabbit inferior vena cava was exposed respectively to PSS, Ca^{2+}-free PSS, 100 µM Mn^{2+} added to Ca^{2+}-free PSS, addition of 25 mM caffeine to the preceding solution, and finally PSS. Caffeine (Caff) caused an initial jump in fluorescence due to its interaction with fura-2 but then decreased the rate of Mn^{2+} quenching of the fura-2 fluorescence. (b) The inferior vena cava was exposed respectively to PSS, Ca^{2+}-free PSS, 100 µM Mn^{2+} added to Ca^{2+}-free PSS and subsequent addition of 30 µM ryanodine (Ryan) to the same solution. Ryanodine did not affect the rate of Mn^{2+} quenching. (c) The protocol was the same as that in (b) with the exception that 2 µM thapsigargin (Thapsig) was added instead of ryanodine. Thapsigargin also had no effect on the rate of Mn^{2+} entry into fura-2 containing cells. (Ordinates indicate fura-2 fluorescence intensities excitation at 360 nm).

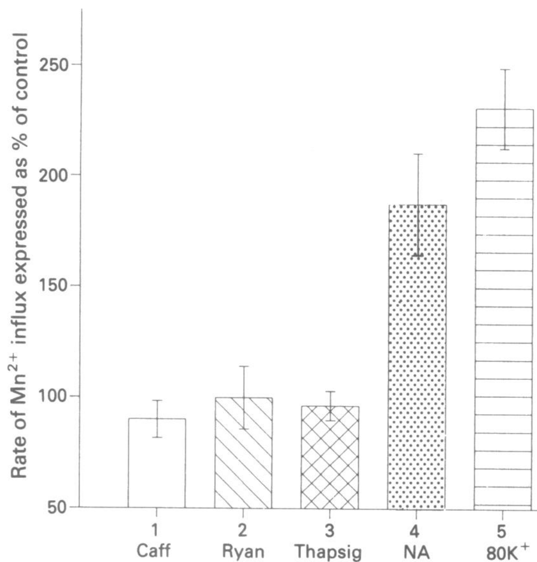
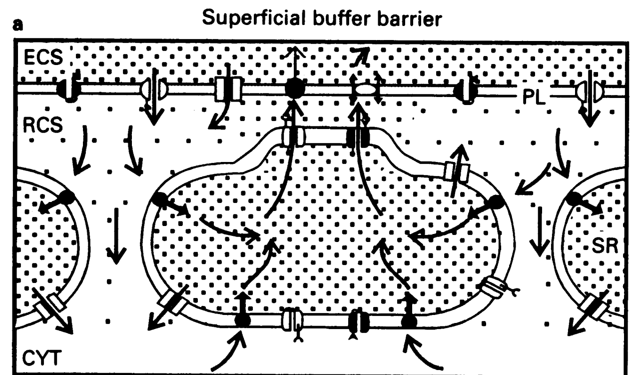


Figure 7 A summary of the effects of different agents and high K^+ solution on the rate of Mn^{2+} influx. The rate of Mn^{2+} influx was measured as described above. Values on the ordinate scale indicate the percentage change in the rate of Mn^{2+} influx compared with respective control group. Each column represents the average of four (five of the experiments with ryanodine) ± s.e. (1) Caff, 25 mM caffeine; (2) Ryan, 30 μM ryanodine; (3) Thapsig, 2 μM thapsigargin; (4) NA, 10 μM noradrenaline; (5) 80 K+, 80 mM K^+ solution. For details, see the legends of Figure 4–Figure 6.

1990). The discrepancy between this conclusion and the one reached in the present paper may be due to the use of widely different preparations and experimental paradigms.

Electron microscopy of native smooth muscle, though showing close junctional areas between plasmalemma and SR, does not support the presence of any type of pore between the ECS and the SR lumen (Devine *et al.*, 1972; Kowarski *et al.*, 1985). The involvement of a restricted cytoplasmic space in the refilling of vascular smooth muscle SR was supported by a delay between the $[Ca^{2+}]_i$ signal and tension development if Ca^{2+} influx was stimulated after the SR had first been depleted (Rembold, 1989; Chen *et al.*, 1992a). This delay, which was abolished by caffeine, demonstrates the ability of the SR Ca^{2+} pump to establish intracellular Ca^{2+} gradients in the smooth muscle cell periphery (Chen *et al.*, 1992a). In agreement with this concept, Benham & Bolton (1986) observed that large numbers of Ca^{2+}-sensitive K^+ channels may be activated in the absence of force development. Recently Sturek and coworkers found that depletion of the SR in bovine aortic cells resulted in a 58% decrease in K^+ current compared to the resting K^+ current, without there being a difference in $[Ca^{2+}]_i$ as measured by fura-2 (Stehno-Bittel & Sturek, 1992). They also showed that unloading of the SR following a period of enhanced Ca^{2+} influx is accompanied by increased activity of Ca^{2+}-activated K^+ channels and a simultaneous decrease in average $[Ca^{2+}]_i$. These electrophysiological data not only provide strong support for the existence of Ca^{2+} gradients near the plasmalemma, but also suggest that SR Ca^{2+} may be released towards the plasmalemma. In other words, there may exist a continuous Ca^{2+} extrusion pathway from the SR lumen to the ECS which involves a transport process in both SR and cell membranes. The function of such a SR unloading mechanism may be to maintain the 'Ca^{2+} buffer barrier' under steady state conditions. Interruption of this process by prevention of SR Ca^{2+} accumulation would lead to an increase in the steady state $[Ca^{2+}]_i$ dependent on the inward Ca^{2+} leak, similar to the results shown in Figure 2 (see below and Figure 8). However an alternative explanation for the maintained elevation of $[Ca^{2+}]_i$ upon discharge of SR Ca^{2+} is provided by Putney's capacitive Ca^{2+} entry model. It states that depletion of an intracellular Ca^{2+} pool leads to opening of plasmalemmal Ca^{2+} channels (Putney, 1990). We tested the possible application of this hypothesis to venous smooth muscle with three different agents which inhibit Ca^{2+} accumulation by the SR in various ways and found that none of them increased the rate of Mn^{2+} entry through the plasmalemma (Figure 6a,b,c). Since we did record Mn^{2+} influx through the Ca^{2+} leak pathway, VGC and ROC (Figure 3a, Figure 4, Figure 5a,b,c), we suggest that the increase in $[Ca^{2+}]_i$ caused by the SR Ca^{2+} discharge cannot be explained by an increase in Ca^{2+} influx from the ECS under our conditions and that NA did not stimulate Ca^{2+} influx by virtue of discharging SR Ca^{2+}.



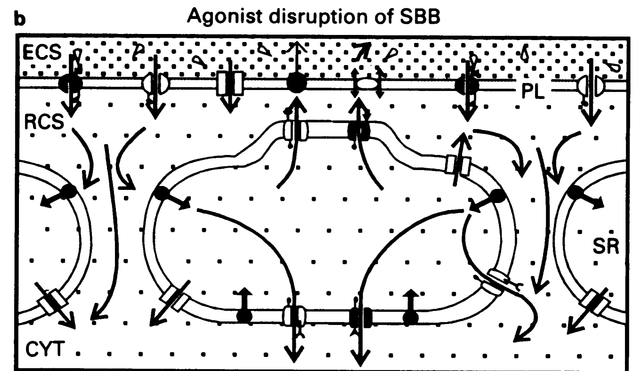


Figure 8 A diagrammatic representation of the Superficial Buffer Barrier (SBB) hypothesis (a) and disruption of the SBB by agonists (b). (a) Ca^{2+} transport in the plasmalemma and the sarcoplasmic reticulum (SR) membrane is mediated by a Ca^{2+} leak, Ca^{2+} channels and Ca^{2+} pumps. In the absence of agonists, Ca^{2+} enters the smooth muscle cell through a non-regulated leak and sporadically opening voltage-gated Ca^{2+} channels (VGC) into a restricted cytoplasmic space near the inner surface of the plasmalemma. A portion of the Ca^{2+} which has entered this space is taken up into the SR by the 100 kD Ca^{2+}-ATPase. The SR therefore buffers Ca^{2+} entry. To maintain this buffer function, SR Ca^{2+} is vectorially released towards the plasmalemma at the plasmalemma-SR junctional regions and extruded into the extracellular space by the 140 kD Ca^{2+}-ATPase and Na^+/Ca^{2+} exchanger (see text). In this model the SR Ca^{2+}-pump assists the plasmalemmal Ca^{2+}-pump in extruding Ca^{2+} from the cells. (b) Agonists stimulate inositol (1,4,5) trisphosphate (IP_3) generation, causing Ca^{2+} release from the SR into the myoplasm short circuiting SBB (see text). The dot density in the aqueous phase represents the $[Ca^{2+}]$. ECS: extracellular space; RCS: restricted cytoplasmic space; CYT: cytoplasm; PL: plasmalemma.

The results displayed in Figure 1 also confirm that the Ca^{2+} content of the SR at rest is well below its maximal capacity (Leijten & van Breemen, 1984), as indicated by the fact that the caffeine-induced $[Ca^{2+}]_i$ transients, which were obtained after Ca^{2+} influx was stimulated, were larger than the first ones obtained after a period of rest. Such would be a requirement for an organelle which at different times functions either as a source of activating Ca^{2+} or as a buffer promoting Ca^{2+} removal from the cytoplasm.

The results presented in this paper: namely, the prevention of SR refilling by thapsigargin and the increase in steady state $[Ca^{2+}]_i$ by this agent, caffeine and ryanodine without an increase in plasmalemmal Ca^{2+} permeability would be best explained by the 'Superficial Buffer Barrier Hypothesis' (see review by van Breemen & Saida, 1989). This hypothesis, which is graphically illustrated in Figure 8a holds that part of the Ca^{2+} which permeates the smooth muscle plasmalemma is taken up by the SR Ca^{2+} pump from a restricted cytoplasmic region before it diffuses into the deeper cytoplasm. In addition it proposes that the buffering is maintained by continuous unloading of the SR to the ECS. As mentioned above, the SR unloading process may involve vectorial Ca^{2+} release towards the inner plasmalemmal surface. A low basal rate of Ins(1,4,5)P_3 synthesis at the plasmalemma combined with an active cytoplasmic Ins(1,4,5)P_3-phosphatase could establish an Ins(1,4,5)P_3 gradient capable of activating only Ins(1,4,5)P_3 receptors on SR membranes in close proximity to the plasmalemma. Ca^{2+} released through the Ins(1,4,5)P_3 activated channels would activate the Ca^{2+}-sensitive channels (ryanodine receptors) to increase further the local $[Ca^{2+}]_i$ in the SR plasmalemmal junctional regions. This local elevation of $[Ca^{2+}]_i$ would facilitate Ca^{2+} extrusion by the Na^+/Ca^{2+} exchanger which is thought to be concentrated in the junctional regions (Moore *et al.*, 1991) as well as by the plasmalemmal Ca^{2+}-ATPase. Further experiments are required to elucidate the precise mechanism of the above SR unloading pathway. When stimulation with agonists keeps the SR Ca^{2+} channels in an open state (Figure 8b), the peripheral SR loses its ability to accumulate Ca^{2+} and therefore to function as a buffer barrier system. Consequently agonists, as well as pharmacological agents which interfere with SR Ca^{2+} accumulation, would abolish the established Ca^{2+} gradient and shunt out the vectorial extrusion pathway.

The above model for the Superficial Buffer Barrier is a specific example of the ways in which closely apposed areas of SR and plasmalemmal membranes, both having an active Ca^{2+} pump and a Ca^{2+} leak, would be able to interact. The resolution of the exact nature of this interaction will depend on the development of new methodology for measuring Ca^{2+} currents and concentrations in highly locallized regions of the smooth muscle cells.

It is tempting to speculate on possible functions of the hypothetical superficial buffer barrier, which was supported by new data in this paper. It clearly represents an additional element of control over intracellular Ca^{2+} signalling in smooth muscle. The buffering of Ca^{2+} entry may merely protect the cells from activation by a relatively large Ca^{2+} leak (due to a high surface to volume ratio of small cells). A more attractive idea is that the superficial buffer barrier enhances the informational content of Ca^{2+} signalling. For example the resulting Ca^{2+} gradients near the inner plasmalemmal surface would allow separate control over different Ca^{2+}-activated mechanisms which are spatially separated, such as Ca^{2+}-activated K^+ channels and protein kinase C located in the cell membrane and myofilaments and their regulatory kinase and phosphatases located in the cytoplasm.

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References

AARONSON, P. & VAN BREEMEN, C. (1981). Effects of Na gradient manipulation upon cellular Ca, ^{45}Ca fluxes and cellular Na in the guinea pig taenia coli. *J. Physiol.*, **319**, 443–461.

BENHAM, C.D. & BOLTON, T.B. (1986). Spontaneous transient outward currents in single visceral and vascular smooth muscle cells of the rabbit. *J. Physiol.*, **381**, 385–406.

BOURREAU, J.-P., ABELA, A.P., KWAN, C.Y. & DANIEL, E.E. (1992). Refilling of acetylcholine-sensitive internal Ca^{2+} store directly involves a dihydropyridine sensitive Ca^{2+}-channel in dog trachea. *Am. J. Physiol.*, (in press).

BRADING, A.F., BURNETT, M. & SNEDDON, P. (1980). The effect of sodium removal on the contractile responses of the guinea-pig taenia coli to carbachol. *J. Physiol.*, **306**, 411–419.

CASTEELS, R. & DROOGMANS, G. (1981). Exchange characteristics of the noradrenaline-sensitive calcium store in vascular smooth muscle cells of rabbit ear artery. *J. Physiol.*, **317**, 263–279.

CHEN, Q., CANNEL, M. & VAN BREEMEN, C. (1992a). The superficial buffer barrier in vascular smooth muscle. *Can. J. Physiol. Pharmacol.*, **70**, 509–514.

CHEN, Q. & VAN BREEMEN, C. (1992b). Function of smooth muscle sarcoplasmic reticulum. In *Inositol Phosphates and Calcium Signalling*. ed. Putney, J.W.Jr. pp. 335–350. New York: Raven Press.

DEVINE, C.E., SOMLYO, A.V. & SOMLYO, A.P. (1972). Sarcoplasmic reticulum and excitation-contraction coupling in mammalian smooth muscles. *J. Cell Biol.*, **52**, 690–718.

EGGERMONT, J.A., VROLIX, M., RAEYMAEKERS, L., WUYTACK, F. & CASTEELS, R. (1988). Ca^{2+} transport ATPase of vascular smooth muscle. *Circ. Res.*, **62**, 266–278.

GOMES DA COSTA, A.G. & MADEIRA, V.M.C. (1986). Magnesium and manganese ions modulate Ca^{2+} uptake and its energetic coupling in sarcoplasmic reticulum. *Arch. Biochem. Biophys.*, **249**, 199–206.

GRYNKIEWICZ, G., POENIE, M. & TSIEN, R.Y. (1985). A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.*, **260**, 3440–3450.

HWANG, K. & VAN BREEMEN, C. (1987). Ryanodine modulation of $^{45}Ca^{2+}$ efflux and tension in rabbit aortic smooth muscle. *Pflügers Arch. Eur. J. Pharmacol.*, **408**, 343–350.

KOWARSKI, D., SHUMAN, H., SOMLYO, A.P. & SOMLYO, A.V. (1985). Calcium release by noradrenaline from central sarcoplasmic reticulum in rabbit main pulmonary artery smooth muscle. *J. Physiol.*, **366**, 153–175.

LEIJTEN, P.A.A. & VAN BREEMEN, C. (1984). The effects of caffeine on the noradrenaline sensitive calcium store in rabbit aorta. *J. Physiol.*, **357**, 327–339.

MEISSNER, G., DARLING, E. & EVELETH, J. (1986). Kinetics of rapid Ca^{2+} release by sarcoplasmic reticulum. Effects of Ca^{2+}, Mg^{2+}, and adenine nucleotides. *Biochemistry*, **25**, 236–244.

MISSIAEN, L., DECLERCK, I., DROOGMANS, G., PLESSERS, L., DE SMEDT, H., RAEYMAEKERS, L. & CASTEELS, R. (1990). Agonist-dependent Ca^{2+} and Mn^{2+} entry dependent on state of refilling of Ca^{2+} stores in aortic smooth muscle cells of the rat. *J. Physiol.*, **427**, 171–186.

MOORE, E.D.W., FOGARTY, K.E. & FAY, F.S. (1991). Role of Na^+/Ca^{2+} exchanger in β-adrenergic relaxation of single smooth muscle cells. *Ann. N.Y. Acad. Sci.*, **639**, 543–549.

NISHIMURA, J., KHALIL, R.A. & VAN BREEMEN, C. (1989). Agonist-induced vascular tone. *Hypertension*, **13**, 835–844.

PUTNEY, J.W.Jr. (1986). A model for receptor-regulated calcium entry. *Cell Calcium*, **7**, 1–12.

PUTNEY, J.W.Jr. (1990). Capacitative calcium entry revisited. *Cell Calcium*, **11**, 611–624.

REMBOLD, C. (1989). Desensitization of swine arterial smooth muscle to transplasmalemmal Ca^{2+} influx. *J. Physiol.*, **416**, 273–290.

ROUSSEAU, E. & MEISSNER, G. (1987). Ryanodine modifies conductance and gating behavior of single Ca^{2+} release channels. *Am. J. Physiol.*, **253**, C364–C368.

ROUSSEAU, E. & MEISSNER, G. (1989). Single cardiac sarcoplasmic Ca^{2+}-release channels: activation by caffeine. *Am. J. Physiol.*, **253**, H328–H333.

SAIDA, K. (1982). Intracellular Ca release in skinned smooth muscle. *J. Gen. Physiol.*, **80**, 191–202.

SAIDA, K. & VAN BREEMEN, C. (1984). Characteristics of the norepinephrine-sensitive Ca^{2+} store in vascular smooth muscle. *Blood Vessels*, **21**, 43–52.

STEHNO-BITTEL, L. & STUREK, M. (1992). Spontaneous sarcoplasm reticulum calcium release and extrusion from bovine, not porcine, coronary artery smooth muscle. *J. Physiol.*, **451**, 49–78.

SUEMATSU, E., HIRATA, M., HASHIMOTO, T. & KURIYAMA, H. (1984). Inositol 1,4,5-trisphosphate releases Ca^{2+} from intracellular store sites in skinned single cells of porcine coronary artery. *Biochem. Biophys. Res. Commun.*, **120**, 481–485.

VAN BREEMEN, C. (1976). Transmembrane calcium transport in vascular smooth muscle. In *Vascular Neuroeffector Mechanism*. ed. Bevan, J.A. pp. 67–79. Basel: S. Karger.

VAN BREEMEN, C. (1977). Calcium requirement for activation of intact aortic smooth muscle. *J. Physiol.*, **272**, 317–329.

VAN BREEMEN, C. & SAIDA, K. (1989). Cellular mechanisms regulating $[Ca^{2+}]_i$ smooth muscle. *Annu. Rev. Physiol.*, **51**, 315–329.

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