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AGONIST-DEPENDENT Ca^{2+} AND Mn^{2+} ENTRY DEPENDENT ON STATE OF FILLING OF Ca^{2+} STORES IN AORTIC SMOOTH MUSCLE CELLS OF THE RAT

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SUMMARY

1. The properties of intracellular Ca^{2+} stores of intact- and of saponin-skinned A7r5 (an established cell line from embryonic rat aorta) smooth muscle cells were studied by measuring $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ fluxes.

2. Application of 5 μM-vasopressin to intact cells increased the fractional loss of $^{45}Ca^{2+}$ in Ca^{2+}-free solution by a factor of 5·2. This effect was not influenced by a pre-incubation with 10 μM-ryanodine. Caffeine (25 mM) did not stimulate the fractional loss of $^{45}Ca^{2+}$ from intact cells.

3. In skinned cells 10 μM-IP_3 (inositol 1,4,5-trisphosphate) and 5 μM-A23187 (a calcium ionophore) released the same amount of $^{45}Ca^{2+}$. This release did not require GTP and was not affected by a pre-incubation with 10 μM-ryanodine. Caffeine (25 mM) did not release stored Ca^{2+}.

4. NaF (1 mM) plus 10 μM-$AlCl_3$ inhibited by 72% the $^{45}Ca^{2+}$ uptake by the IP_3-sensitive store of skinned cells at 0·15 μM-Ca^{2+}. Cyclic AMP-dependent protein kinase did not stimulate this ATP-dependent $^{45}Ca^{2+}$ uptake, nor could the presence of phospholamban be demonstrated immunologically.

5. The $^{45}Ca^{2+}$ uptake by cells which had been depleted of Ca^{2+} with 5 μM-vasopressin was 69% higher than the uptake obtained without such preceding depletion. This enhanced $^{45}Ca^{2+}$ uptake did not occur through voltage-operated Ca^{2+} channels, because blockade of these channels with verapamil, or depolarization of the plasma membrane by increasing $[K^+]$ from 5·9 to 59 mM in the presence of verapamil, did not modify this uptake.

6. A similar increase of the $^{54}Mn^{2+}$ uptake occurred in intact cells with a depleted Ca^{2+} store. If, however, the cells were first skinned and subsequently exposed to $^{54}Mn^{2+}$, the ATP-dependent $^{54}Mn^{2+}$ uptake amounted to less than 6% of the ATP-dependent $^{45}Ca^{2+}$ uptake.

7. If intact cells were first exposed to a $^{45}Ca^{2+}$- or $^{54}Mn^{2+}$-containing solution, and subsequently skinned in a non-radioactive intracellular solution, the addition of

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10 μM-A23187 to these cells released stored Ca^{2+} or Mn^{2+}. The amount of released Ca^{2+} was only slightly larger than the amount of released Mn^{2+}. If the intracellular store was depleted before loading, the amount of Ca^{2+} or Mn^{2+} released by the ionophore increased by 68 and 28%, respectively.

8. It is concluded that A7r5 smooth muscle cells do not express a Ca^{2+}-induced Ca^{2+} release mechanism, but do contain an IP_3-induced Ca^{2+} release mechanism which can release approximately all intracellularly accumulated $^{45}Ca^{2+}$. Furthermore, the state of filling of this IP_3-sensitive Ca^{2+} store regulates the entry of Ca^{2+} and Mn^{2+}. Possible pathways for such agonist-dependent divalent cation entry are discussed.

INTRODUCTION

Changes in the concentration of free intracellular Ca^{2+} play a predominant role in the regulation of the contraction–relaxation cycle in smooth muscle. This activator Ca^{2+} is either released from intracellular stores (mainly the endoplasmic reticulum) or represents extracellular Ca^{2+} which enters the cell through Ca^{2+} channels in the plasma membrane. The Ca^{2+} can be released from the endoplasmic reticulum of smooth muscle cells, either by a Ca^{2+}-induced Ca^{2+} release mechanism, or by IP_3. Their respective Ca^{2+} release channels seem to be different entities (Ehrlich & Watras, 1988). In most tissues only part of the endoplasmic reticulum is sensitive to inositol 1,4,5-trisphosphate (IP_3) and mostly only about one-third of the Ca^{2+} accumulated by the endoplasmic reticulum can be released by IP_3 (Berridge, 1988; Berridge & Irvine, 1989).

The first aim of the present experiments was to characterize the release of $^{45}Ca^{2+}$ from intracellular stores in A7r5 cells, an established smooth muscle cell line from embryonic rat aorta, and to compare the release of $^{45}Ca^{2+}$ from saponin-treated cells with $^{45}Ca^{2+}$ fluxes of intact cells. Up till now there has been no information reported on the properties of intracellular Ca^{2+} stores in these cells, although A7r5 cells have often been used to study the regulation of intracellular Ca^{2+} (Doyle & Rüegg, 1985; Rüegg, Doyle, Zuber & Hof, 1985; Galizzi, Qart, Fosset, van Renterghem & Lazdunski, 1987; Sperti & Colucci, 1987; Inoue & Kawashima, 1988; Kawashima, 1988; Van Renterghem, Romey & Lazdunski, 1988; Vigne, Breittmayer, Lazdunski & Frelin, 1988*a*; Vigne, Breittmayer, Duval, Frelin & Lazdunski, 1988*b*; Vigne, Breittmayer, Frelin & Lazdunski, 1988*c*; Siskind, McCoy, Chobanian & Schwartz, 1989). Evidence will be presented that A7r5 cells do not contain a Ca^{2+}-induced Ca^{2+} release mechanism and that IP_3 can release approximately all accumulated Ca^{2+} from the Ca^{2+} store.

A second part of this study concerns entry of Ca^{2+} through a pathway which is modulated by the degree of filling of the intracellular store. Models for Ca^{2+} entry dependent on the extent of filling of the intracellular Ca^{2+} store have been proposed (Casteels & Droogmans, 1981; Putney, 1986; Rink & Hallam, 1989). However, the apparent communication between the plasma membrane and the endoplasmic reticulum remains unclear. A direct channel-like connection between the store and the extracellular space has been proposed for smooth muscle (Casteels & Droogmans, 1981), in which couplings between endoplasmic reticulum and plasma membrane have been demonstrated (Devine, Somlyo & Somlyo, 1972). Another possibility is

that the plasma membrane and the endoplasmic reticulum are closely apposed and that Ca^{2+} entering the cell would be pumped into this superficial endoplasmic reticulum. The Ca^{2+} entry into the cell would be increased if IP_3 releases Ca^{2+} from this store (the capacitative model proposed by Putney, 1986). Recent experiments from the latter group, however, led to the suggestion that this capacitative model might be partially incorrect (Takemura, Hughes, Thastrup & Putney, 1989). Their experiments, based on the action of thapsigargin, a substance that releases Ca^{2+} by inhibiting the endoplasmic reticulum Ca^{2+} pump, seem to suggest that the permeability of the plasma membrane at some distance from the store is controlled by the extent of filling of the store. Refilling would occur via an influx into the cytoplasm followed by an active uptake into the store. We have followed a different approach to determine the involvement of a Ca^{2+} pump in the agonist-dependent increase in Ca^{2+} entry by analysing $^{54}Mn^{2+}$ fluxes. Mn^{2+} entry dependent on the extent of filling of the intracellular store has been reported in human platelets and neutrophils (Merritt, Jacob & Hallam, 1989; Sage, Merritt, Hallam & Rink, 1989). Micromolar concentrations of Mn^{2+} are not transported by the endoplasmic reticulum Ca^{2+}-transporting ATPases (Gomes da Costa & Madeira, 1986) and Mn^{2+} can therefore be used to discriminate between the several models of agonist-dependent Ca^{2+} entry. We observed that a depletion of the Ca^{2+} store stimulates the Ca^{2+} and Mn^{2+} entry into these A7r5 cells. The $^{54}Mn^{2+}$ fluxes are suggestive of the existence of a pathway that follows a direct connection between the plasma membrane and the endoplasmic reticulum, as originally proposed by Casteels & Droogmans (1981).

METHODS

Cell culture

A7r5 cells from the American Tissue Type Culture Collection CRL 1446 (Bethesda, MD, USA) were obtained through Flow Laboratories (Irvine, Scotland). The cells were cultured at 37 °C in a 9% CO_2 incubator in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 3·8 mM-L-glutamine, 0·9% (v/v) non-essential amino acids, 85 IU ml^{-1} penicilline and 85 μg ml^{-1} streptomycin. The cells were subcultured weekly by trypsinization. They were seeded in twelve-well clusters (Costar, Ma, USA, 3·8 cm^2) at a density of approximately 15000 cells cm^{-2}. Experiments were carried out with confluent monolayers of cells between the 5th and 9th day after plating.

$^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ fluxes

For $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ fluxes, the wells were fixed on a plate with a thermostat (either at 35 or 25 °C) and placed on a mechanical shaker. In a first step, the culture medium was aspirated and the monolayers were equilibrated for 1 h with a modified Krebs solution of the following composition (mM): 135 NaCl; 5·9 KCl; 1·5 $CaCl_2$; 1·2 $MgCl_2$; 11·6 HEPES and 11·5 glucose (pH 7·3).

For experiments on intact cells, the uptake of $^{45}Ca^{2+}$ was initiated by adding a 0·2 or 1·5 mM-$^{45}Ca^{2+}$-containing modified Krebs solution at 35 °C to the cells for a period of 5 min. The uptake of $^{54}Mn^{2+}$ was measured for the same period of time using a modified Krebs solution without $CaCl_2$ and containing 1·5 mM-$MnCl_2$. The uptake process was efficiently stopped by aspirating the radioactive medium and washing the monolayers twice with an ice-cold Ca^{2+}-free solution containing 2 mM-EGTA. Afterwards, 1 ml of Ca^{2+}-free 2 mM-EGTA-containing Krebs solution at 35 °C was added to the monolayers and replaced every 2 min over a 30 min period. At the end of the efflux, the monolayers were solubilized in 2% sodium dodecyl sulphate (SDS). The $^{45}Ca^{2+}$ present in each of the effluent samples and the remaining radioactivity in the cells were measured in a liquid scintillation counter using Instagel II (Packard, Zürich, Switzerland). The amount of $^{54}Mn^{2+}$ in the effluent

samples and that remaining in the cells at the end of the efflux were counted in a γ-counter. The time course of the tracer wash-out was calculated by summing in retrograde order the amount of tracer remaining in the cells at the end of the efflux and the amounts of tracer collected during the successive time intervals. This time course became monoexponential after about 20 min and the amplitude of this slowly exchanging fraction was used to estimate the cellular $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ content (Fig. 1).

Skinning was obtained by incubating for 10 min at 25 °C with 20 μg ml^{-1} of saponin in the following skinning solution (mM): 100 KCl; 30 imidazole; 2 $MgCl_2$; 1 ATP and 1 EGTA (pH 7·0). The cells were subsequently loaded for 5 min at 25 °C with $^{45}Ca^{2+}$ by exposure to a medium containing (mM): 100 KCl; 30 imidazole; 5 $MgCl_2$; 5 ATP; 0·44 EGTA; 5 NaN_3 and 0·12 $CaCl_2$ (pH 7·0). The free Ca^{2+} concentration in this solution was 0·15 μM. The efflux was performed exactly as described for experiments on intact cells, except that the efflux medium had the following composition (mM): 100 KCl; 30 imidazole; 2 $MgCl_2$; 1 ATP; 1 EGTA and 5 NaN_3 (pH 7·0). These experiments were performed at 25 °C because skinned cells deteriorate more rapidly at higher temperatures (Itoh, Kuriyama & Suzuki, 1981). In these experiments where $^{45}Ca^{2+}$ uptake and $^{54}Mn^{2+}$ uptake by skinned cells were compared, a free concentration of 0·2 and 10 μM of either of these ions, in the presence and absence of ATP, was used. The total concentration of Ca^{2+}, Mn^{2+} and Mg^{2+} was calculated using the following logarithms of the association constants: H-ATP, 6·49; H-HATP, 4·11; Ca-ATP, 3·78; Ca-HATP, 1·98; Mg-ATP, 4·00; Mg-HATP, 2·06; Mn-ATP, 4·76; Mn-HATP, 2·39; H-EGTA, 9·47; H-HEGTA, 8·85; Ca-EGTA, 10·97; Ca-HEGTA, 5·33; Mg-EGTA, 5·21; Mg-HEGTA, 3·37; Mn-EGTA, 12·18; Mn-HEGTA, 6·88.

In the experiment represented in Fig. 5 and Table 3, intact cells were first loaded with $^{45}Ca^{2+}$ or $^{54}Mn^{2+}$ for 5 min at 37 °C. The efflux was performed as described above, but using the following intracellular solution at 25 °C (mM): 100 KCl; 30 imidazole; 2 $MgCl_2$; 1 ATP; 1 EGTA and 5 NaN_3 (pH 7·0). This solution was supplemented with 20 μg ml^{-1} of saponin for the first 10 min, in order to skin the cells during the efflux procedure.

ELISA assay (enzyme-linked immunosorbent assay)

KCl-extracted microsomes (microsomal fractions) from pig stomach smooth muscle and from A7r5 cells were prepared as described by Wuytack, De Schutter & Casteels (1981). The membranes (0–100 μg ml^{-1}) were diluted in a sodium bicarbonate buffer (15 mM-Na_2CO_3–35 mM-$NaHCO_3$), pH 9·8, and coated in ninety-six well immunoplates (Nunc, Denmark) at 100 μl well^{-1} by incubating overnight at 4 °C. Unbound antigens were removed by washing three times with phosphate-buffered saline. Incubation with a mouse monoclonal antibody against phospholamban was performed for 3 h at 37 °C. Unbound antibodies were removed by washing three times as mentioned above. Rabbit anti-mouse immunoglobulines conjugated with horseradish peroxidase (Dakopatts, Denmark) were used as second step reagents. After washing three times, the absorbance at 490 nm was measured using H_2O_2 as the substrate and ortho-phenylene diamine as the detection system.

Drugs

The catalytic subunit of cyclic AMP-dependent protein kinase from bovine heart (P2645), IP_3, vasopressin and saponin were from Sigma (St Louis, MO, USA). The catalytic subunit was restored to activity in 50 mg ml^{-1} dithiothreitol, pH 7·4 (0·5 mg protein ml^{-1}) The ionophore A23187 was received as a free acid from Calbiochemicals (San Diego, CA, USA). ATP was from Boehringer (Mannheim, FRG). Ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) was from Fluka (Switzerland) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulphonic acid (HEPES) from Merck (Darmstadt, FRG). Ryanodine was from S. B. Penick Co. (New York, NY, USA). $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ were from Amersham International (UK). All other reagents were of the highest chemical purity commercially available.

Statistics

Unless otherwise stated, all values are given as means ± S.E.M.

RESULTS

Effect of vasopressin and caffeine on stored Ca^{2+} in intact cells

The application of vasopressin to intact A7r5 cells induces IP_3 production (Doyle & Rüegg, 1985). It was therefore of interest to examine the effect of 5 μM-vasopressin

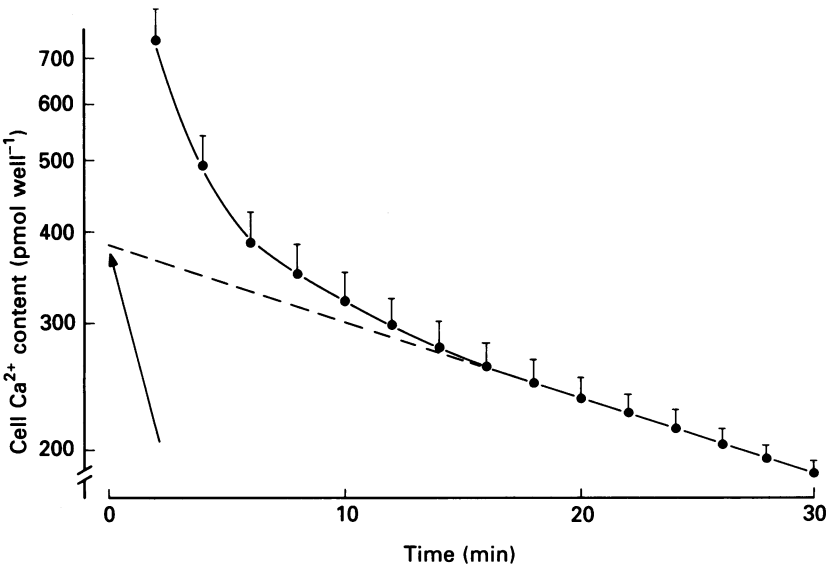

Fig. 1. The efflux of $^{45}Ca^{2+}$ from cells loaded for 5 min in a solution containing 0·2 mM-$^{45}Ca^{2+}$. The Ca^{2+} content (expressed on a logarithmic scale as pmol Ca^{2+} $well^{-1}$) is represented as a function of time of efflux in Ca^{2+}-free medium. The slowly declining phase of the Ca^{2+} content of the cells can be extrapolated to time 0 as indicated by the dashed line. This extrapolated value represents the estimated value of the cellular Ca^{2+} content at the start of the efflux. The curve represents mean ± S.E.M. of six wells.

on the fractional loss of $^{45}Ca^{2+}$ in Ca^{2+}-free solution. The fractional loss represents the amount of $^{45}Ca^{2+}$ leaving the cell, normalized to the amount of labelled $^{45}Ca^{2+}$ present in the cell at that time. Figure 2*A* illustrates that vasopressin increased the fractional loss of $^{45}Ca^{2+}$ from 0·05 ± 0·01 ($n = 7$) to 0·26 ± 0·01 ($n = 7$) per minute. This effect could be ascribed to a release of stored $^{45}Ca^{2+}$ and an ensuing increased Ca^{2+} extrusion from the cell. A second application of vasopressin was without effect, indicating that the application of this high dose of vasopressin depleted the IP_3-sensitive intracellular Ca^{2+} store. Pre-incubation of the cells for 1 h with 10 μM-ryanodine in the 1·5 mM-Ca^{2+}-containing modified Krebs solution, before loading them with $^{45}Ca^{2+}$, did not affect the stimulation of the $^{45}Ca^{2+}$ efflux induced by vasopressin (Fig. 2*B*). This finding indicates that ryanodine did not deplete the vasopressin-sensitive Ca^{2+} store. In parallel experiments and using the same solutions, ryanodine depleted the noradrenaline-sensitive Ca^{2+} store in strips of the rabbit ear artery, as described previously (Kanmura, Missiaen, Raeymaekers & Casteels, 1988). In contrast with the observations on primary cultures of rat aorta (Kanaide, Shogakiuchi & Nakamura, 1987), we could not observe an increase of the fractional loss of $^{45}Ca^{2+}$ from A7r5 cells during exposure to 25 mM-caffeine (Fig. 2*C*). In addition, the vasopressin-induced

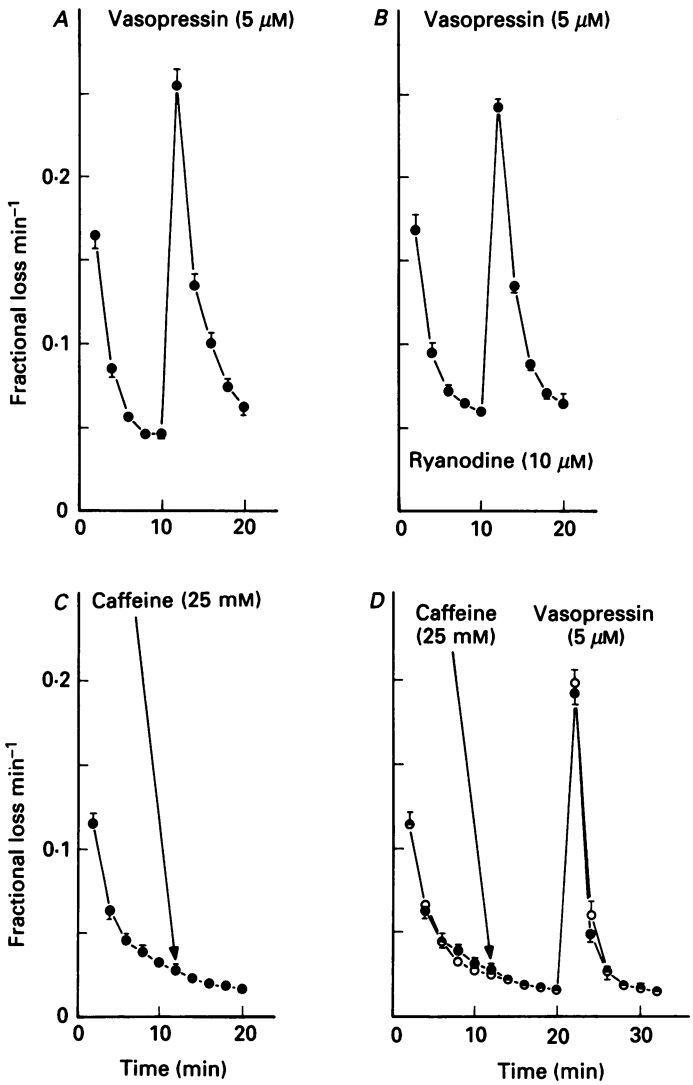

Fig. 2. Effect of vasopressin and caffeine on the fractional loss of $^{45}Ca^{2+}$ in Ca^{2+}-free solution. *A* represents the effect of 5 μM-vasopressin added for a 2 min period, 10 min after starting the efflux, *B* that of vasopressin in the presence of 10 μM-ryanodine and *C* that of 25 mM-caffeine. *D* shows the effect of 5 μM-vasopressin applied 20 min after starting the efflux under control conditions (○) and following the application of 25 mM-caffeine after 10 min of efflux (●). Each curve represents the mean ± S.E.M. of six wells.

increase of the fractional $^{45}Ca^{2+}$ loss was not affected by a preceding treatment with caffeine (Fig. 2*D*). This finding again indicates that caffeine does not release any stored $^{45}Ca^{2+}$ from intact cells.

Effects of IP_3 and caffeine in saponin-skinned smooth muscle cells

Figure 3 represents the effects of presumed Ca^{2+}-releasing agents on saponin-skinned smooth muscle cells. After 10 min incubation in the non-radioactive solution, 10 μM-IP_3 increased this fractional loss from 0·04 ± 0·01 ($n = 4$) to 0·20 ± 0·01

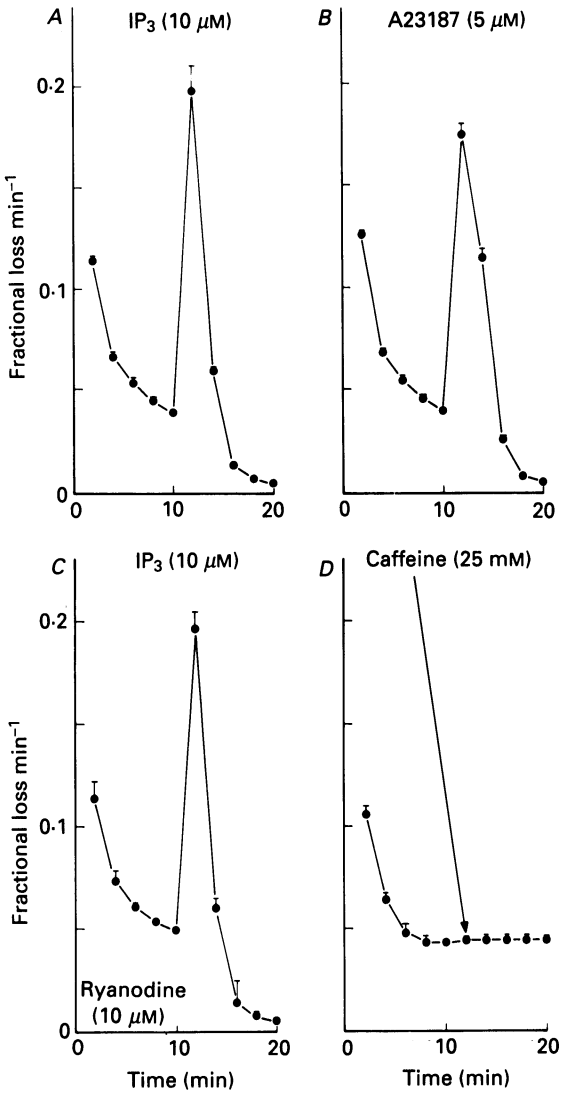

Fig. 3. Effect of presumed Ca^{2+} releasers on Ca^{2+} stores in saponin-skinned cells. The effects of 10 μM-IP_3 (*A*), 5 μM-A23187 (*B*) and of 25 mM-caffeine (*D*), all applied for a 2 min period 10 min after starting the $^{45}Ca^{2+}$ efflux, are represented. *C* represents the effect of 10 μM-IP_3 after pre-incubation of the cells with 10 μM-ryanodine. Each curve represents the mean $\pm$ S.E.M. of four wells.

($n = 4$) per minute (Fig. 3*A*). This effect was observed in a medium without added GTP. A23187 (5 μM) enhanced the fractional loss of $^{45}Ca^{2+}$ to a similar extent as IP_3 (Fig. 3*B*), suggesting that IP_3 released almost 100% of all accumulated $^{45}Ca^{2+}$. Application of A23187 after stimulation of the cells with 10 μM-IP_3 did not increase this fractional loss (results not shown).

Pre-incubation of intact cells for 1 h with 10 μM-ryanodine, before the skinning procedure, did not affect the subsequent IP_3-induced increase of the fractional loss of $^{45}Ca^{2+}$ (Fig. 3*C*). For these experiments ryanodine was present during the skinning

procedure, during the loading of the store with $^{45}Ca^{2+}$ and during the efflux procedure. This finding confirms our experiments on intact cells indicating that ryanodine did not deplete the IP_3-sensitive Ca^{2+} store in A7r5 smooth muscle cells. The application of 25 mM-caffeine to the skinned cells did not increase the fractional loss of $^{45}Ca^{2+}$ (Fig. 3*D*), as has also been observed for intact cells.

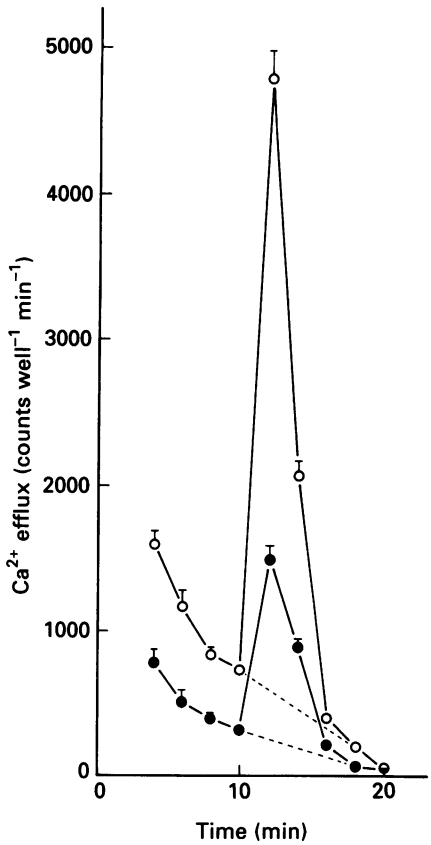

Fig. 4. Effect of fluoroaluminates on the ATP-dependent $^{45}Ca^{2+}$ uptake in saponin-skinned cells as estimated from the change of the rate of efflux (in counts $well^{-1}$ min^{-1}) induced by 5 μM-A23187. The efflux from cells loaded in the presence of 1 mM-NaF and 10 μM-$AlCl_3$ is represented by ● while the efflux from cells loaded in the absence of the fluoroaluminate complex is represented by ○. The efflux was performed in the absence of the fluoroaluminate complex. Each curve represents the mean ± S.E.M. of four wells.

Effects of fluoroaluminates on the loading of stores in skinned cells

AlF_4^- has been reported to inhibit 'P'-type cation transport ATPases, such as that of the endoplasmic reticulum Ca^{2+} pump (Missiaen, Wuytack, De Smedt, Vrolix & Casteels, 1988; Missiaen, Wuytack, De Smedt, Amant & Casteels, 1989). The effect of adding 1 mM-NaF and 10 μM-$AlCl_3$ to the solution during the period of ATP-dependent accumulation of $^{45}Ca^{2+}$ in the store could be assessed by measuring the amount of $^{45}Ca^{2+}$ released by 5 μM-A23187 (Fig. 4). At a free Ca^{2+} concentration of 0·15 μM, fluoroaluminates inhibit the accumulation of Ca^{2+} in the store by 72%. A

higher concentration of NaF could not be used, because the solubility product of MgF_2 would be exceeded. Although we therefore do not know whether the inhibition of the Ca^{2+} pump is complete, this experiment certainly indicates, that, at least in the presence of this low Ca^{2+} concentration of 0·15 μM, most of the Ca^{2+} was taken up via the endoplasmic reticulum Ca^{2+} pump.

The ATP-dependent $^{45}Ca^{2+}$ accumulation is not regulated by phospholamban

After skinning, A7r5 cells were exposed for 30 min to 50 nM of the catalytic subunit of cyclic AMP-dependent protein kinase. The reason for the long incubation time was that phosphorylation of the plasma membrane Ca^{2+} pump by this kinase was relatively slow and reached a plateau only after 45 min (Neyses, Reinlib & Carafoli, 1985). Thereupon the ATP-dependent $^{45}Ca^{2+}$ uptake was measured by monitoring the amount of $^{45}Ca^{2+}$ released by 10 μM-IP_3. This amount of released $^{45}Ca^{2+}$ after pre-incubation with the kinase was $96 \pm 6\%$ ($n = 3$) of that observed without pre-incubation, indicating that cyclic AMP-dependent protein kinase does not stimulate the ATP-dependent $^{45}Ca^{2+}$ accumulation in these cells.

Since cyclic AMP-dependent protein kinase interacts with the Ca^{2+} pump via phosphorylation of phospholamban, we have tried to demonstrate the presence of phospholamban immunologically. No positive signal for phospholamban could be obtained in the KCl-extracted microsomal fraction from A7r5 cells. The same ELISA applied to a microsomal fraction from stomach smooth muscle prepared by the same procedure and to a sarcoplasmic reticulum fraction from pig heart yielded a positive reaction.

Depletion of the Ca^{2+} store promotes Ca^{2+} entry via a pathway different from voltage-operated Ca^{2+} channels

The $^{45}Ca^{2+}$ uptake was measured in cells with a filled agonist-sensitive Ca^{2+} store and in cells in which this store was emptied. The following protocol was used to deplete the intracellular Ca^{2+} store: the cells were first prepared for 2 min with a Ca^{2+}-free and 2 mM-EGTA-containing modified Krebs solution, after which 5 μM-vasopressin was added for 2 min in order to deplete the store (Fig. 2*A*). These cells with an empty store were finally exposed for 2 min to a Ca^{2+}-free and 2 mM-EGTA-containing modified Krebs solution supplemented with 10 μM-verapamil. Cells with a filled store were obtained by pre-incubating the cells with the same solutions, except that the second solution did not contain vasopressin.

The cellular $^{45}Ca^{2+}$ uptake in the presence of 10 μM-verapamil by cells with a filled and by those with an empty Ca^{2+} store are given for different concentrations of Ca^{2+} in Table 1. The difference between the values observed in these two cell populations represents the Ca^{2+} uptake induced by prior depletion of the store. Emptying the store enhanced the $^{45}Ca^{2+}$ uptake by 120 pmol $well^{-1}$ 5 min^{-1} at 1·5 mM-Ca^{2+}. This enhanced Ca^{2+} uptake was less pronounced at 0·2 mM-Ca^{2+}. Essentially the same enhancing effect on the $^{45}Ca^{2+}$ uptake was obtained when the incubation period in Ca^{2+}-free solution after the vasopressin pulse was extended from 2 min to 2 h before starting the $^{45}Ca^{2+}$ uptake.

There are two arguments against the possibility that the increased $^{45}Ca^{2+}$ uptake by cells with a depleted Ca^{2+} store occurred through voltage-dependent Ca^{2+}

TABLE 1. Uptake of $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ at 37 °C by A7r5 cells from solutions containing the indicated K^+ and Ca^{2+} or Mn^{2+} concentrations

	0·2 mM-Ca^{2+}		1·5 mM-Ca^{2+}	1·5 mM-Mn^{2+}
	(5·9 mM-K^+)	(59 mM-K^+)	(5·9 mM-K^+)	(5·9 mM-K^+)
Filled store	69±4 (7)	71±3 (3)	319±5 (3)	290±12 (6)
Empty store	117±8 (7)	124±7 (3)	439±2 (3)	429±10 (6)
Stimulation	48±12 (7)	53±10 (3)	120±7 (3)	139±22 (6)

Cells with an empty Ca^{2+} store were obtained by pre-incubation with 5 μM-vasopressin in Ca^{2+}-free solution. Cells with a filled Ca^{2+} store were subjected to the same procedure but in the absence of vasopressin. The uptake media contained 10 μM-verapamil and the cells were exposed to these solutions for 5 min. The difference between the values obtained on cells with a filled and empty store represents the stimulation of the uptake induced by emptying the intracellular Ca^{2+} store. The cellular contents were determined as indicated in the legend of Fig. 1 and are expressed as means±S.E.M. (pmol well^{-1} 5 min^{-1}) for the number of observations given in parentheses.

TABLE 2. ATP-dependent $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ uptake at 25 °C from solutions containing 0·2 or 10 μM-free Ca^{2+} or Mn^{2+} by saponin-skinned A7r5 cells

Concentration	$^{45}Ca^{2+}$ uptake	$^{54}Mn^{2+}$ uptake
0·2 μM	328±51 (3)	6±3 (4)
10 μM	636±86 (4)	39±5 (2)

The values represent the uptake in the presence of 5 mM-ATP, corrected for the passive binding of the cation which was measured in the absence of 5 mM-ATP. The results (pmol well^{-1} 5 min^{-1}) are expressed as means±S.E.M. and the number of observations is given in parentheses.

channels. The first argument is that these experiments were done in the presence of 10 μM-verapamil. This concentration of verapamil indeed completely inhibited the voltage-dependent Ca^{2+} channels, because the Ca^{2+} uptake at 0·2 mM-Ca^{2+} and 5·9 mM-K^+ (69±4 pmol well^{-1} 5 min^{-1}, $n = 7$) was not different from that at 0·2 mM-Ca^{2+} and 59 mM-K^+ (71±3 pmol well^{-1} 5 min^{-1}, $n = 3$), while in the absence of verapamil the latter $^{45}Ca^{2+}$ uptake was increased by a factor of 3 (authors' unpublished observation). A second argument is that the enhancement of the $^{45}Ca^{2+}$ uptake induced by emptying the store was not increased by depolarizing the membrane (48±12 pmol well^{-1} 5 min^{-1} ($n = 7$) at 5·9 mM-K^+ and 53±10 pmol well^{-1} 5 min^{-1} ($n = 3$) at 59 mM-K^+).

Depletion of the Ca^{2+} store promotes Mn^{2+} entry

A7r5 cells can take up $^{54}Mn^{2+}$ from a Krebs solution in which 1·5 mM-$CaCl_2$ is replaced by 1·5 mM-$MnCl_2$ (Table 1). The $^{54}Mn^{2+}$ uptake was significantly increased if the intracellular Ca^{2+} store was previously depleted. This enhancement was very similar to that observed for the $^{45}Ca^{2+}$ uptake. The increased $^{54}Mn^{2+}$ uptake in cells with a depleted Ca^{2+} store did not occur through voltage-dependent Ca^{2+} channels, because these experiments were also done in the presence of 10 μM-verapamil.

Next we investigated whether $^{54}Mn^{2+}$ is a substrate for the endoplasmic reticulum Ca^{2+} pump. We therefore studied the ATP-dependent $^{45}Ca^{2+}$ and $^{54}Mn^{2+}$ uptake in saponin-skinned cells at a free concentration of 0·2 and 10 μM of these cations (Table 2). In contrast to the Ca^{2+} uptake, skinned cells did not significantly accumulate Mn^{2+}, even at 10 μM. At this Mn^{2+} concentration the Mn^{2+} uptake amounted to only

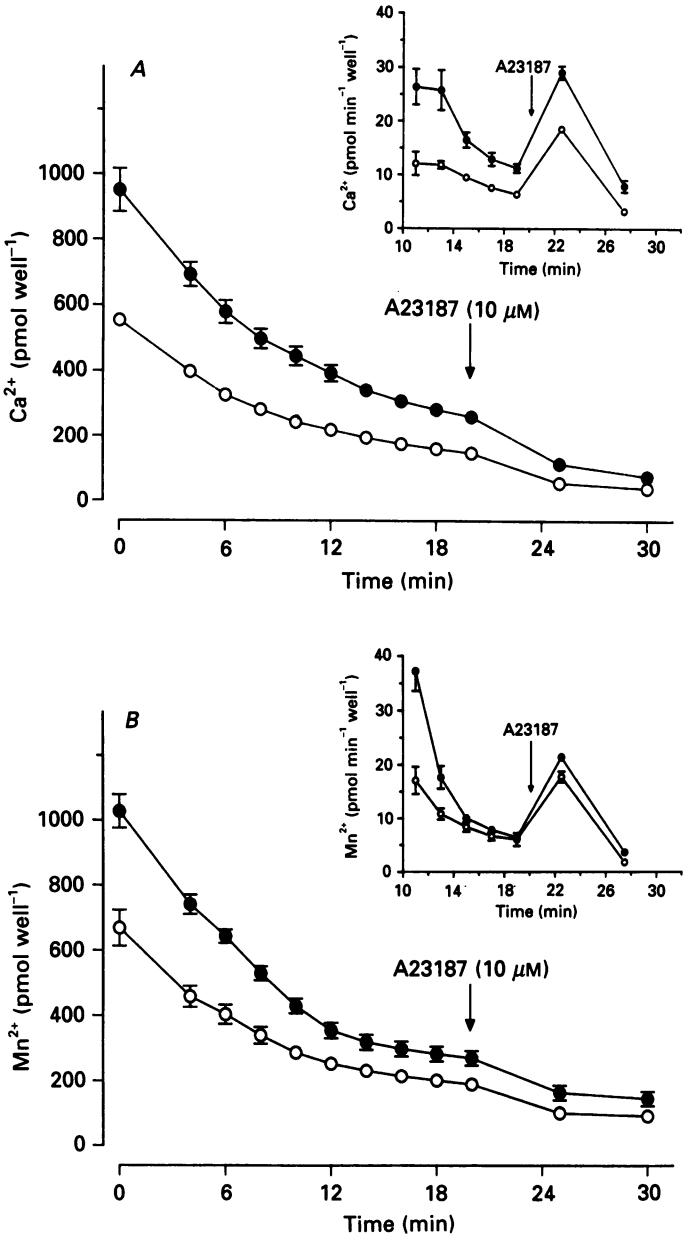

Fig. 5. *A*, A23187-induced Ca^{2+} release from the store after loading intact cells with 1·5 mM-$^{45}Ca^{2+}$ followed by the skinning procedure. ○ represent the efflux curves from cells that were loaded with a filled store. ● represent efflux curves from cells in which the store was emptied prior to the loading procedure. The efflux medium had an intracellular composition and was supplemented with 20 μg ml^{-1} saponin for the first 10 min (see Methods). The tracing represents the decrease of Ca^{2+} (pmol well^{-1}) on a linear scale, as a function of time of efflux. The inset represents the rate of efflux (pmol min^{-1} well^{-1}) as a function of time. The concentration of A23187 was 10 μM. Each curve represents the mean ± S.E.M. of six wells. *B* represents a similar experiment after loading with 1·5 mM-$^{54}Mn^{2+}$.

6% of the ATP-dependent Ca^{2+} uptake. The latter finding implies that Mn^{2+}, compared with Ca^{2+}, is a poor substrate for the endoplasmic reticulum Ca^{2+} pump, as has also been observed for the Ca^{2+} pump of skeletal muscle sarcoplasmic reticulum (Gomes da Costa & Madeira, 1986) and of liver endoplasmic reticulum (Joseph & Williamson, 1986).

We next tried to establish whether the increased cellular $^{54}Mn^{2+}$ uptake observed after depleting the store was accompanied by an increased uptake of $^{54}Mn^{2+}$ in the store. The experimental procedure consisted of loading intact cells with an emptied or filled store in a 1·5 mM-$^{54}Mn^{2+}$-containing solution for 5 min, followed by skinning the cells and subsequently adding 10 μM-A23187 to these skinned cells in order to evaluate the fraction of Mn^{2+} which could be released from the stores (see Methods). The reason for application of A23187 rather than IP_3 for measuring the amount of Mn^{2+} in the store was that it is not known whether IP_3 can release Mn^{2+} from the stores. Figure 5 illustrates the efflux of $^{45}Ca^{2+}$ or $^{54}Mn^{2+}$ after loading intact cells with an empty or filled store in a solution containing 1·5 mM-$^{45}Ca^{2+}$ or $^{54}Mn^{2+}$ respectively. This efflux was performed in a medium of intracellular composition, to which 20 $\mu g\ ml^{-1}$ of saponin was added during the first 10 min to skin the cells. After 20 min, the efflux was continued for another 10 min in the presence of 10 μM-A23187. A striking finding was that the ionophore could release an appreciable amount of $^{54}Mn^{2+}$, 89% of the amount of $^{45}Ca^{2+}$ released (Table 3). A23187 released 68% more labelled Ca^{2+} from cells with a depleted store prior to Ca^{2+} loading than from the control cells (Table 3). In cells which had been loaded with $^{54}Mn^{2+}$ after depletion of the store, 28% more $^{54}Mn^{2+}$ was released during exposure to A23187, as compared to controls. The amount of labelled $^{45}Ca^{2+}$ or $^{54}Mn^{2+}$ remaining in the skinned cells after 10 min of ionophore treatment was considered as a non-releasable fraction and is also given in Table 3. In both experimental conditions (empty or filled store), the non-releasable part of $^{54}Mn^{2+}$ was larger than that of $^{45}Ca^{2+}$. This could be due to a higher affinity of Mn^{2+} for binding sites within or outside the store or to a less efficient transfer of Mn^{2+} through the A23187-stimulated pathway.

DISCUSSION

Calcium-mobilizing agonists, such as vasopressin, release Ca^{2+} from those intracellular stores in smooth muscle that are responsible for part of the pharmacomechanical coupling (Somlyo & Somlyo, 1968; Droogmans, Raeymaekers & Casteels, 1977). It was recently observed that vasopressin increased the production of IP_3 in A7r5 cells (Doyle & Rüegg, 1985). We now report that IP_3 can release Ca^{2+} from an intracellular compartment in these cells, most probably the endoplasmic reticulum. In most cell types investigated so far, IP_3 released only a fraction of the Ca^{2+} pool that can be released by A23187 (Berridge, 1988). This finding is often considered to indicate that the endoplasmic reticulum is composed of at least two compartments: an IP_3-sensitive and an IP_3-insensitive one. In this study using A7r5 smooth muscle cells we observed that 10 μM-IP_3 and 5 μM-A23187 released the same amount of $^{45}Ca^{2+}$, indicating that IP_3 released virtually all accumulated Ca^{2+} and that GTP was not necessary to obtain a complete release of Ca^{2+} by IP_3. This finding suggests that the Ca^{2+} store in these cells represents one single compartment.

Besides an IP_3-induced Ca^{2+} release, many muscle cells also contain a Ca^{2+}-induced Ca^{2+} release mechanism. The latter release occurs through a channel that is distinct from the IP_3 release channel (Ehrlich & Watras, 1988). This Ca^{2+}-induced Ca^{2+} release channel can be activated by caffeine (Liu, Lai, Rousseau, Jones & Meissner, 1989) and is permanently opened by ryanodine into a state of reduced conductance

TABLE 3. Releasable and non-releasable Ca^{2+} and Mn^{2+} taken up by intact cells during a loading procedure with an empty or filled store

	Ca^{2+} uptake		Mn^{2+} uptake	
	Releasable	Non-releasable	Releasable	Non-releasable
Filled store	109±3 (6)	39±5 (6)	97±5 (6)	92±14 (6)
Empty store	185±9 (6)	75±7 (6)	125±4 (6)	146±22 (6)

The experiments were performed as described in the legend of Fig. 5. The amount of releasable Ca^{2+} and Mn^{2+} in the store was estimated from the release induced by 10 μM-A23187 from the skinned cells over a 10 min period after 20 min of efflux. The amount of non-releasable Ca^{2+} and Mn^{2+} was determined from the amount of the tracer left in the cells after application of the ionophore. The results (pmol $well^{-1}$) are expressed as means ± S.E.M. for the number of observations given in parentheses. For clarity, the absolute values of these results cannot be compared with those in Table 1, which represent values extrapolated to time 0. Furthermore, these experiments were performed on a different batch of cells compared to those described in Table 1.

(Fill & Coronado, 1988). The treatment of intact or skinned smooth muscle strips with ryanodine in the presence of Ca^{2+} will therefore deplete this intracellular Ca^{2+} store (Hwang & van Breemen, 1987; Kanmura *et al.* 1988) However, the findings that in A7r5 cells caffeine did not release stored Ca^{2+} and that ryanodine did not reduce the amount of Ca^{2+} in the store both indicate that A7r5 cells do not have a functional Ca^{2+}-induced Ca^{2+} release mechanism.

The ATP-dependent accumulation of $^{45}Ca^{2+}$ in the IP_3-sensitive compartment was decreased in skinned cells by up to 72% by fluoroaluminates. Fluoroaluminates are reported to inhibit several 'P'-type cation transport ATPases (Missiaen *et al.* 1988). The oxalate-stimulated ATP-dependent $^{45}Ca^{2+}$ uptake is decreased by fluoroaluminates in a crude microsomal fraction of visceral smooth muscle, and since the endoplasmic reticulum is permeable to oxalate, this was an indication, but no proof, that the endoplasmic reticulum Ca^{2+} pump could also be inhibited by fluoroaluminates (Missiaen *et al.* 1989). The present findings provide more direct evidence of the inhibition of the ATP-dependent accumulation of $^{45}Ca^{2+}$ in an IP_3-sensitive compartment in skinned cells and of the inhibition *in situ* of the activity of the endoplasmic-reticulum (Ca^{2+}–Mg^{2+})-ATPases by fluoroaluminates.

Phospholamban, a protein which is associated with the endoplasmic reticulum Ca^{2+} pump in cardiac muscle, is also present in smooth muscle (Raeymaekers & Jones, 1986; Eggermont, Vrolix, Raeymaekers, Wuytack & Casteels, 1988). Its phosphorylation by cyclic AMP-, cyclic GMP- and Ca^{2+}-calmodulin-dependent protein kinase and by protein kinase C is responsible for stimulation of the Ca^{2+} pump. The ATP-dependent Ca^{2+} uptake was not stimulated by cyclic AMP-dependent protein kinase in A7r5 cells. Furthermore, the presence of phospholamban could not be demonstrated immunologically. These results therefore suggest that the

Ca^{2+} pump in these smooth muscle cells is possibly not affected by regulatory proteins.

The hypothesis that agonists stimulate Mn^{2+} entry into human platelets, neutrophils and endothelial cells through a pathway unrelated to voltage-dependent Ca^{2+} channels has been supported by measuring the quenching of the fluorescence of Fura-2. This increased Mn^{2+} influx occurred in platelets with depleted intracellular stores, suggesting that the state of filling of the stores might influence bivalent cation entry across the plasma membrane (Hallam, 1987; Merritt *et al.* 1989; Sage *et al.* 1989; Thastrup, Dawson, Scharff, Foder, Cullen, Drobak, Bjerrum, Christensen & Hanley, 1989). Our results, obtained with a different approach, i.e. measuring the $^{54}Mn^{2+}$ uptake into cells, confirm that depleting the agonist-sensitive intracellular store enhanced the $^{54}Mn^{2+}$ uptake through a verapamil-insensitive pathway. This enhancing effect of the Mn^{2+} uptake into intact cells was of similar magnitude as the increase of the Ca^{2+} entry induced by depleting the Ca^{2+}-store. There is now a general consensus that depletion of the IP_3-sensitive Ca^{2+} pool activates a process for rapid refilling of the store via an increased Ca^{2+} influx across the plasma membrane. Different models for such Ca^{2+} entry, dependent on the state of filling of the intracellular Ca^{2+} store, have been proposed (Casteels & Droogmans, 1981; Putney, 1986; Takemura *et al.* 1989), but the exact description of the connection between the plasma membrane and the endoplasmic reticulum remains speculative. The increased divalent cation entry could occur directly into the store (Casteels & Droogmans, 1981), into a narrow space of the cytoplasm between the store and the plasma membrane (Putney, 1986) or into the bulk of the cytoplasm (Takemura *et al.* 1989). Our results with $^{54}Mn^{2+}$ fluxes show: (1) Mn^{2+} is a poor substrate for the endoplasmic reticulum Ca^{2+} pump. (2) Both Ca^{2+} and Mn^{2+} are equally well accumulated within the intracellular store in cells with a filled store. (3) The entry of Mn^{2+} into intact cells after depletion of the store is increased to the same extent as that of Ca^{2+}. (4) Depletion of the Ca^{2+} store prior to the loading significantly increased the amount of releasable Mn^{2+} in the store, but this increase is smaller than for Ca^{2+}. The observations that Mn^{2+}, a poor substrate for the endoplasmic reticulum Ca^{2+} pump, is equally as well accumulated as Ca^{2+} into the store in cells with a filled store, and that its uptake is increased after depleting the store, are compatible with a direct filling of the store from the extracellular compartment. This conclusion is mainly based on the finding that at a concentration of 10 μM-free Mn^{2+}, there is virtually no ATP-dependent uptake of Mn^{2+} in skinned cells. The other proposed pathways would therefore require much higher levels of free Mn^{2+}, which in view of the high buffering capacity of the cytosol (Von Tscharner, Deranbeau & Baggiolini, 1986) is very unlikely. Filling of the store by ATP-dependent uptake via the cytosol seems therefore improbable.

In conclusion, A7r5 smooth muscle cells do not express a Ca^{2+}-induced Ca^{2+} release mechanism, but do contain an IP_3-induced Ca^{2+} release mechanism that releases mainly all accumulated Ca^{2+}. Their endoplasmic reticulum therefore contains a remarkably simplified Ca^{2+} release mechanism. No evidence for a modulation of the endoplasmic reticulum Ca^{2+} pump by phospholamban could be demonstrated. The Mn^{2+} and Ca^{2+} influx is increased after depleting the agonist-sensitive store. The observation that a considerable fraction of Mn^{2+} is found in an A23187-releasable

store suggest that it may have reached that location by direct filling from the extracellular compartment.

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