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Vasopressin stimulation of Ca^{2+} mobilization, two bivalent cation entry pathways and Ca^{2+} efflux in A7r5 rat smooth muscle cells

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1. Arg^8-vasopressin (AVP)-regulated Ca^{2+} transport pathways were investigated in fura-2-loaded A7r5 cells using both single cell and population measurements.

2. AVP evokes an initial concentration-dependent rise in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) to a peak which is independent of extracellular Ca^{2+}, and a sustained Ca^{2+} signal that results from a balance between stimulation of Ca^{2+} entry and efflux.

3. Depletion of intracellular Ca^{2+} stores with thapsigargin, ionomycin, or prior treatment with AVP in Ca^{2+}-free medium activates 'capacitative' entry of Ca^{2+}, Ba^{2+} or Mn^{2+}. Capacitative Mn^{2+} entry is inhibited by refilling stores with Ca^{2+}; neither Sr^{2+} nor Ba^{2+} substitute for Ca^{2+} to give this effect.

4. In cells with empty stores, AVP stimulates further bivalent cation entry, and the effect persists when extracellular Na^+ is replaced by *N*-methyl-D-glucamine or under depolarizing conditions (extracellular KCl concentration ($[KCl]_o$), 135 mM). This effect of AVP is not therefore merely a consequence of AVP causing membrane hyperpolarization or stimulation of Na^+–Ca^{2+} exchange, but results from opening of a bivalent cation influx pathway.

5. Several lines of evidence indicate that AVP-stimulated bivalent cation entry is not a consequence of more complete emptying of the intracellular stores and consequent further activation of the capacitative pathway. AVP stimulates Ba^{2+} entry when the intracellular Ca^{2+} stores have been both emptied by ionomycin and prevented from refilling by thapsigargin. Mn^{2+} permeates the capacitative pathway, but AVP does not further increase Mn^{2+} entry, confirming that AVP does not further activate the capacitative pathway and that the two pathways differ in their permeability to Mn^{2+}. When the extracellular $[Sr^{2+}]$ is low, empty stores do not stimulate detectable Sr^{2+} entry, but addition of AVP causes substantial Sr^{2+} entry.

6. A decrease in $[Ca^{2+}]_i$ occurs when 50 nM AVP is added during a sustained elevation of $[Ca^{2+}]_i$ evoked by thapsigargin. Since AVP does not inhibit the capacitative pathway, this result suggests that AVP stimulates Ca^{2+} extrusion.

7. We conclude that stimulation of Ca^{2+} mobilization, two modes of bivalent cation entry, and Ca^{2+} efflux all contribute to the complex concentration-dependent effects of AVP in A7r5 smooth muscle cells.

Many cells respond to extracellular stimuli with an increase in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) that usually results from changes in Ca^{2+} transport across both the plasma membrane and the membranes of intracellular Ca^{2+} stores. The importance of the phosphoinositide pathway in controlling these Ca^{2+} fluxes is established (Berridge, 1993). Receptor activation stimulates phosphoinositidase C-catalysed hydrolysis of phosphatidyl-inositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate (InsP_3) and diacylglycerol. InsP_3 binds to its receptor in the endoplasmic reticulum, opens the intrinsic Ca^{2+} channel of that receptor and thereby stimulates Ca^{2+} mobilization. Diacylglycerol and Ca^{2+} together activate protein kinase C.

Sustained responses to hormones are maintained by stimulation of Ca^{2+} entry across the plasma membrane. This process generally involves Ca^{2+} entry through voltage-insensitive pathways and is believed to be important for the

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refilling of intracellular Ca^{2+} stores. One such pathway, which is activated by hormones via depletion of intracellular Ca^{2+} stores, has been termed the 'capacitative' Ca^{2+} entry pathway (Putney, 1986). Activation of this pathway by empty stores requires neither continued receptor occupation nor elevated levels of inositol phosphates (Putney, 1990). Electrophysiological recordings from mast cells have identified a Ca^{2+} current that is activated by depletion of intracellular Ca^{2+} stores and is highly selective for Ca^{2+} over other cations (Hoth & Penner, 1992, 1993); it may therefore represent the activity of the capacitative Ca^{2+} entry pathway. Inositol 1,3,4,5-tetrakisphosphate (InsP_4), the immediate product of InsP_3 phosphorylation, has also been reported to activate Ca^{2+} entry (Irvine & Cullen, 1993), although the evidence remains controversial (Bird, Rossier, Hughes, Shears, Armstrong & Putney, 1991). Electrophysiological evidence in support of this pathway has come from endothelial cells where InsP_4 or agonists that stimulate phosphoinositide hydrolysis activate channels that appear to be similarly permeable to Mn^{2+}, Ca^{2+} and Ba^{2+} (Lückhoff & Clapham, 1992). These studies and others (Felder, Poulter & Wess, 1992; Clementi, Scheer, Zacchetti, Fasolato, Pozzan & Meldolesi, 1992) suggest that hormonal stimulation of cells may activate more than one Ca^{2+} entry pathway, and that these pathways may be distinguishable by their permeability to different divalent cations. The present study exploits these features to examine the effects of Arg^8-vasopressin (AVP) on Ca^{2+} entry and efflux pathways in fura-2-loaded A7r5 cells, a vascular smooth muscle cell line (Kimes & Brandt, 1976).

A preliminary account of some of our results has been published (Taylor & Byron, 1994).

METHODS

Cell culture and fura-2 loading

A7r5 cells were cultured as described previously (Byron & Taylor, 1993). For fura-2 loading, cells on coverslips were washed twice with control medium (composition (mM): 135 NaCl, 5·9 KCl, 1·5 $CaCl_2$, 1·2 $MgCl_2$, 11·5 glucose, 11·6 4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid (Hepes), pH 7·3) and then incubated in the same medium with 2 μM of the acetoxymethyl ester form of fura-2 (fura-2 AM), 0·1% (w/v) BSA, and 0·025% (v/v) Pluronic F127 detergent for 90–120 min at 20–23 °C. After loading, the cells were washed twice and incubated in control medium for a further 1–5 h. This final incubation allowed complete hydrolysis of fura-2 AM, as assessed by the shift in the fluorescence excitation spectrum. About 95% of the fura-2 was released from the cells within 3 min of addition of saponin (50 μg ml^{-1}), suggesting that about 95% of the dye was in the cytosol; subsequent experiments (see Discussion) further confirmed that most of the indicator was cytosolic.

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) measurements

For measurements of $[Ca^{2+}]_i$ in populations of cells, a rectangular coverslip with a confluent monolayer of fura-2-loaded cells was mounted vertically in a 4·5 ml optical methacrylate cuvette containing 2·5 ml of control medium. The cuvette was placed in a Perkin-Elmer LS50B (Beaconsfield, UK) or Hitachi F4500 (Tokyo, Japan) spectrofluorimeter with the coverslip at a 30 deg angle to the excitation light path. Cells were excited for 0·25 s at 0·1–0·2 s intervals and the emission at 510 nm was collected for analysis. Excitation wavelengths (λ_{ex}) are specified in the figure legends. Autofluorescence of cells (< 10% of the fluorescence from fura-2-loaded cells) did not vary with time and was not, therefore, subtracted from single wavelength recordings from cell populations. In some experiments using the Perkin-Elmer LS50B instrument, cells were excited alternately with 340 and 380 nm light every 0·02 s using a rotating filter wheel in the path of the excitation light. An integrated ratio ($\lambda_{ex,340\ nm}/\lambda_{ex,380\ nm}$) of the light emitted at 510 nm was then determined at 1 s intervals.

The solution bathing the cells was changed by perfusing fresh solution from gravity-fed reservoirs into the bottom of the cuvette while aspirating continuously from just above the coverslip. At the perfusion rates used (5–10 ml min^{-1}), the half-time for mixing in the cuvette was approximately 25 s and complete exchange occurred within 50 s; faster perfusion rates dislodged the cells. Figure labels indicate when the solutions were switched, rather than the times when complete exchange of the medium was accomplished. All experiments were conducted in the presence of either nimodipine (50 nM) or verapamil (10 μM) to block voltage-gated L-type Ca^{2+} channels, which we previously showed to be responsible for spontaneous Ca^{2+} spiking in A7r5 cells (Byron & Taylor, 1993). Similar results were obtained in the presence of either antagonist, and neither antagonist affected the Ca^{2+} mobilization evoked by a maximal concentration of AVP (not shown).

For measurements of $[Ca^{2+}]_i$ in single cells, fura-2-loaded cells on a round coverslip were mounted in a perfusion chamber, covered with control medium and placed on the stage of a Nikon Diaphot inverted epifluorescence microscope. In these single cell experiments, complete exchange of solutions in the perfusion chamber was accomplished in 10–20 s. Alternating excitation light was provided by computer-controlled switching of narrow band interference filters in front of a 100 W xenon arc lamp. The emitted fluorescence passed through a dichroic mirror (400 nm) and high-pass barrier filter (480 nm) and was directed to an intensified CCD video camera (Photonic Science, Milham, UK). Video images captured approximately every 600 ms were digitized and stored in the memory of an Applied Imaging Magiscan image analyser. Image analysis was performed as described previously (Byron & Taylor, 1993).

Calibration of fluorescence signals

Fura-2–Ca^{2+} fluorescence signals were calibrated using standard solutions as described previously (Byron & Villereal, 1989). Ca^{2+}, Sr^{2+} and Ba^{2+} produce similar changes in fura-2 fluorescence when excited with 340 or 380 nm light, but the different bivalent cations differ in their affinities for fura-2 (at 20 °C, dissociation constant (K_D) values are 227, 7600 and 1360 nM, respectively; Kwan & Putney, 1990) and in their isosbestic excitation wavelengths (360, 364 and 370 nm, respectively; Byron & Taylor, 1993). We have not attempted in our studies of either single cells or cell populations to calibrate the fluorescence signals recorded in the presence of extracellular Sr^{2+} or Ba^{2+} because it is impossible to determine the contribution of the remaining Ca^{2+} to the fluorescence signal. Instead, these results are presented with the same calibration scales used to report $[Ca^{2+}]_i$, thereby allowing both evaluation of the Ca^{2+} signal prior to addition of Sr^{2+} or Ba^{2+} and quantitative comparison of the magnitudes of the fluorescence signals.

Materials

Cell culture media were from Gibco BRL. Fura-2 AM, fura-2 pentapotassium salt and Pluronic F127 were from Molecular Probes, Inc. EGTA (puriss grade) was from Fluka Chemical Co., Gillingham, UK. Ionomycin and Hepes were from Calbiochem. Nimodipine was from Cookson Chemicals (Southampton, UK). [1-(β-mercapto-β,β,-pentamethylene-propionic acid)-2-O-methyl tyrosine]arginine-vasopressin ($d(CH_2)_5[Tyr(CH_3)^2,Ala-NH_2{}^9]$-AVP) was a gift from Dr M. Manning (Medical College of Ohio, Toledo, OH, USA). All other chemicals, including AVP, thapsigargin, N-methyl-D-glucamine (NMDG) and verapamil, were from Sigma.

Ca^{2+}-free media were prepared by omitting $CaCl_2$ from the control media and supplementing them with EGTA (0·1–1 mM). Media that included Ba^{2+} or Sr^{2+} were supplemented with EGTA to minimize the effects of contaminating Ca^{2+}; the final free Ba^{2+} and Sr^{2+} concentrations may be estimated from the total [$BaCl_2$] or [$SrCl_2$] minus the concentration of EGTA added. The figure legends indicate the total concentrations of bivalent cations and EGTA added.

Results are given as means ± S.E.M.

RESULTS

Concentration-dependent effects of AVP on $[Ca^{2+}]_i$

In populations of A7r5 cells, both the magnitude and shape of the Ca^{2+} signal varied with the concentration of AVP. These concentration-dependent effects of AVP on the $[Ca^{2+}]_i$ signals recorded from cell populations do not reflect recruitment of a greater proportion of cells as the concentration of AVP is increased because, from analyses of single cells, even a very low concentration of AVP (0·25 nM) evoked substantial increases in $[Ca^{2+}]_i$ in all cells (about 75 cells from 2 independent experiments). At the lowest AVP concentrations (≤ 0·25 nM), $[Ca^{2+}]_i$ slowly increased after a substantial latency (≤ 170 s) to a modestly elevated level (≤ 50 nM above the original baseline) that was sustained for more than 10 min (Fig. 1). Higher concentrations of AVP caused $[Ca^{2+}]_i$ to rise after a much shorter latency to a peak which increased with AVP concentration. The sustained phase of the response decreased at the higher AVP concentrations, such that a maximal concentration of AVP (50 nM) evoked a rapid, transient elevation of $[Ca^{2+}]_i$ followed by a slower decline to below the original baseline (Fig. 1). The peak increases in $[Ca^{2+}]_i$ result largely from mobilization of intracellular Ca^{2+} stores because neither their amplitude nor their sensitivity to AVP were affected by omission of Ca^{2+} from the incubation media (not shown). In the absence of extracellular Ca^{2+}, the increase in $[Ca^{2+}]_i$ was transient and was followed by a rapid decline to below the original baseline (by ≤ 20 nM; the extent of the decline increased as the concentration of AVP increased), suggesting activation of a Ca^{2+} efflux pathway. In the presence of extracellular Ca^{2+}, the sustained responses to AVP were more complex: the highest concentrations of AVP (≥ 25 nM) caused $[Ca^{2+}]_i$ to fall to below the original baseline, whereas after stimulation with lower concentrations of AVP, $[Ca^{2+}]_i$ remained elevated for at least 10 min (Fig. 1). The sustained response to AVP

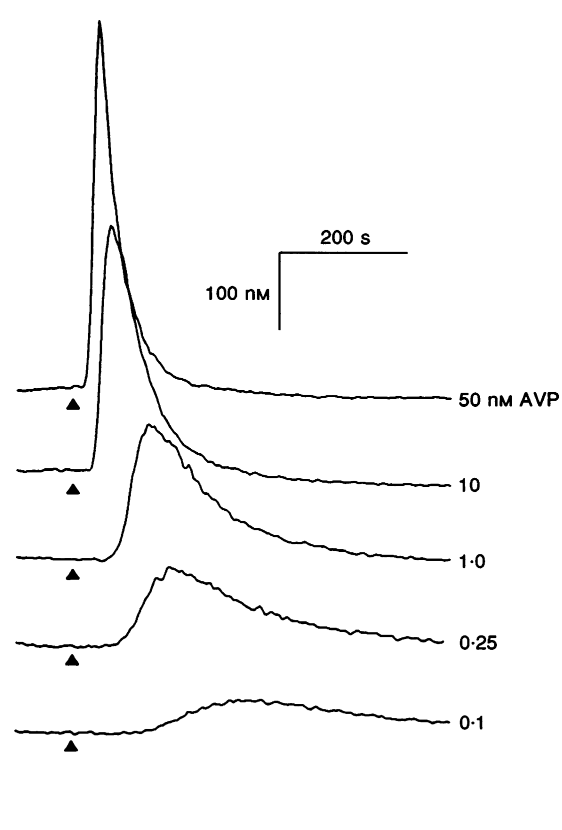

Figure 1. Concentration-dependent effects of AVP on $[Ca^{2+}]_i$ in A7r5 cells

Representative traces (typical of results from 24 coverslips) drawn to the same scale from populations of cells stimulated with the indicated concentrations of AVP in medium containing Ca^{2+} and verapamil (10 μM). Arrowheads denote the onset of perfusion of the cuvette with AVP.

therefore appears to result from a combination of its effects on Ca^{2+} influx and efflux pathways. In subsequent experiments, we have examined the mechanisms underlying these effects of AVP.

Capacitative Ca^{2+} entry

In many cells, depletion of intracellular Ca^{2+} stores is associated with activation of a capacitative Ca^{2+} entry pathway (Putney, 1990). Thapsigargin, a selective inhibitor of the Ca^{2+}-ATPases of the endoplasmic reticulum (Thastrup, Cullen, Drobak, Hanley & Dawson, 1990), was used to empty the intracellular Ca^{2+} stores of A7r5 cells and reveal whether such a capacitative Ca^{2+} entry mechanism exists in these cells. Thapsigargin (500 nM) evoked a biphasic elevation of $[Ca^{2+}]_i$ to a plateau of about 400 nM that was sustained for as long as extracellular Ca^{2+} was present (Fig. 2*A*). The elevated $[Ca^{2+}]_i$ rapidly returned to its basal level after removal of extracellular Ca^{2+}, and restoration of extracellular Ca^{2+} to cells treated with thapsigargin in Ca^{2+}-free medium resulted in a rapid rise in $[Ca^{2+}]_i$ to about 400 nM (Fig. 2*B*). Similar results were recorded from single A7r5 cells (Fig. 8*B*).

Under conditions in which addition of Ca^{2+} (1·5 mM) to the extracellular medium caused substantial capacitative Ca^{2+} entry, there was no detectable change in fura-2 fluorescence when Sr^{2+} (1·5 mM) rather than Ca^{2+} was added to the medium (Fig. 2). Capacitative Ba^{2+} (1·5 mM) entry, in contrast, was detectable under these conditions (see below).

In order to examine whether Mn^{2+} permeated the capacitative entry pathway, a protocol was employed in which intracellular stores were emptied either by transient exposure of the cells to AVP (50 nM for 250 s) in Ca^{2+}-free medium, or by prolonged treatment with thapsigargin (500 nM for 30 min) in Ca^{2+}-free medium. The latter treatment completely emptied the intracellular stores since subsequent addition of ionomycin (1 μM) failed to evoke further detectable Ca^{2+} mobilization (not shown). After the AVP treatment, about 30–50% of the intracellular Ca^{2+} stores remained and could be emptied by addition of thapsigargin or ionomycin (not shown). The effects of emptying the intracellular Ca^{2+} stores on Mn^{2+} entry were determined by measuring the quenching of fura-2 fluorescence ($\lambda_{ex} = 360$ nm) following addition of $MnCl_2$ (0·5 mM) to the extracellular medium. Basal rates of Mn^{2+} entry differed between experiments; we therefore established the basal rate at the beginning of each experiment by exposing cells briefly (150 s) to extracellular $MnCl_2$ (0·5 mM) and recording the rate of fluorescence quench during the last 50 s of the Mn^{2+} exposure. For each experiment, all subsequent measurements of Mn^{2+} quench were expressed relative to this basal rate. Depletion of the intracellular Ca^{2+} stores with either AVP or thapsigargin significantly enhanced the rate of Mn^{2+} entry ($P < 0.05$) compared with unstimulated control cells incubated for the same period in Ca^{2+}-free medium. The effects of thapsigargin and prior treatment with AVP were

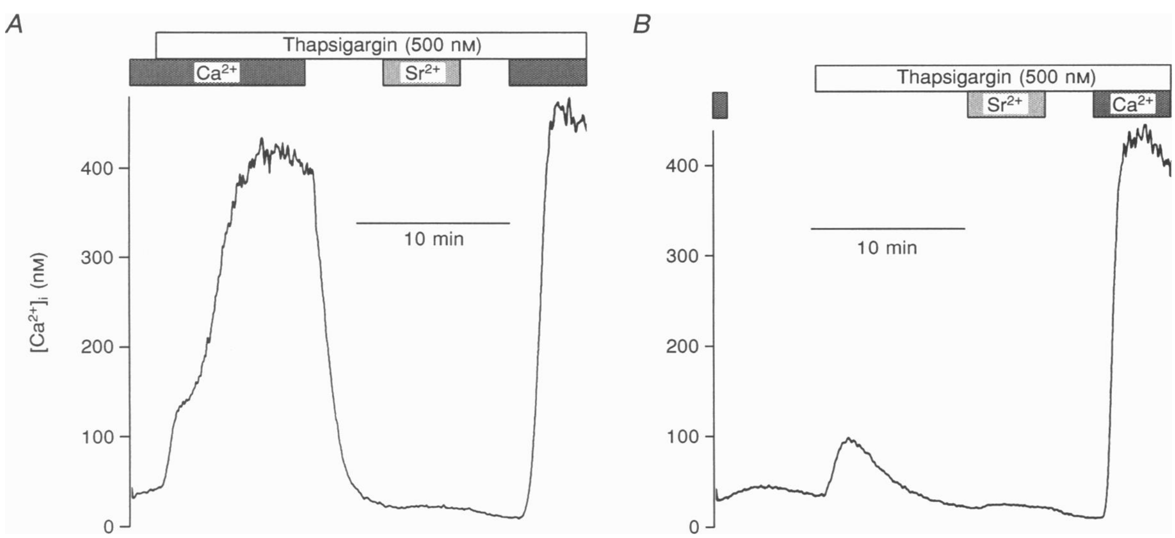

Figure 2. Effects of thapsigargin on Ca^{2+} mobilization and Ca^{2+} entry

Representative traces from cell populations, each typical of at least 3 similar experiments, showing the effects of thapsigargin when applied in Ca^{2+}-containing (*A*) or Ca^{2+}-free (+ 0·1 mM EGTA, *B*) medium. Switches to media containing thapsigargin (500 nM, open bars), extracellular Ca^{2+} (1·5 mM + 0·1 mM EGTA, dark grey bars) or extracellular Sr^{2+} (2·5 mM + 1 mM EGTA, light grey bars) were made at the times shown. Verapamil (10 μM) was included in all media. Similar results were observed in measurements from single cells (not shown).

quantitatively similar: the rates of Mn^{2+} quench, relative to the basal rates, were increased by $(12{\cdot}5 \pm 3{\cdot}8)$-fold (AVP, $n = 5$) and $(13{\cdot}5 \pm 1{\cdot}8)$-fold (thapsigargin, $n = 3$), respectively, whereas Mn^{2+} entry to control cells was increased by only $(5{\cdot}1 \pm 0{\cdot}9)$-fold ($n = 8$) (Fig. 3).

To establish that the enhanced rate of Mn^{2+} entry was a consequence of depletion of intracellular Ca^{2+} stores, cells in which the stores had been emptied by AVP were exposed transiently to medium containing extracellular Ca^{2+} to allow the stores to refill with Ca^{2+}. A 5 min exposure to medium containing either 1·5 or 10 mM Ca^{2+} fully reversed the stimulatory effects of prior exposure to AVP on Mn^{2+} entry (Fig. 3): the rates of Mn^{2+} entry fell from $(12{\cdot}5 \pm 3{\cdot}8)$-fold ($n = 5$) to $(3{\cdot}5 \pm 1{\cdot}5)$-fold ($n = 4$) of the basal rates in the cells with previously emptied stores, and from $(5{\cdot}1 \pm 0{\cdot}9)$-fold to $(3{\cdot}5 \pm 1{\cdot}5)$-fold ($n = 4$) in control cells. An increase in Mn^{2+} permeability of about 3-fold during the 40 min incubation cannot be attributed to depletion of intracellular Ca^{2+} stores because a similar increase was observed in untreated cells incubated for the same time in Ca^{2+}-containing medium (not shown). The reversal of the effects of prior exposure to AVP is unlikely to result from non-specific effects of exposure to extracellular Ca^{2+} because in thapsigargin-treated cells, which are unable to refill their intracellular stores, transient exposure (5 min) to medium containing 10 mM $CaCl_2$ had no effect on Mn^{2+} entry (Fig. 3). Neither $BaCl_2$ (10 mM) nor $SrCl_2$ (10 mM) were able to effectively substitute for $CaCl_2$ during the refilling period and reduce the rate of Mn^{2+} entry (Fig 3*D* and *E*).

A second bivalent cation entry pathway activated by AVP

Several protocols were employed to determine whether capacitative Ca^{2+} entry can wholly account for the stimulatory effects of AVP on Ca^{2+} entry. In the first series of experiments, the Ca^{2+} ionophore ionomycin was used to empty intracellular Ca^{2+} stores. Ionomycin, because it selectively transports Ca^{2+}, but not Sr^{2+} or Ba^{2+}, across biological membranes (Liu & Hermann, 1978), can be used to deplete intracellular Ca^{2+} stores without directly affecting Sr^{2+} or Ba^{2+} transport (Byron & Taylor, 1993). Incubation of cells with ionomycin (1 μM for 5 min) in Ca^{2+}-free medium effectively emptied the intracellular stores because subsequent addition of either AVP (50 nM) or thapsigargin (500 nM) failed to evoke detectable Ca^{2+} mobilization (not shown). Addition of Sr^{2+} (1·5 mM) to the medium bathing these cells did not produce a detectable change in fura-2 fluorescence (Fig. 4), confirming our earlier observation that depletion of stores by thapsigargin failed to evoke detectable Sr^{2+} entry (Fig. 2). However, subsequent addition of AVP (10 nM) evoked a sustained elevation of fura-2 fluorescence (Fig 4*A* and *B*) that rapidly reversed after removal of extracellular Sr^{2+} (Fig. 4*A*). Both this effect of AVP and AVP-stimulated Ca^{2+} mobilization were abolished in the presence of $d(CH_2)_5[Tyr(CH_3)^2,Ala\text{-}NH_2^{\,9}]$-AVP (100 nM) (not shown), a selective peptide antagonist of V_{1a}-vasopressin receptors (Manning, Stoev, Bankowski, Misicka & Lammek, 1992). Addition of AVP (10 nM) after removal of extracellular Sr^{2+} evoked a small transient increase in fluorescence (Fig. 4*C*), suggesting that part of the initial Sr^{2+} signal following addition of AVP resulted from mobilization of Sr^{2+} from intracellular stores.

Addition of extracellular Ba^{2+} (0·5–3 mM) to cells in Ca^{2+}-free medium caused a gradual increase in the fura-2 fluorescence signal ($\lambda_{ex} = 360$ nm) that increased with increasing Ba^{2+} concentration (not shown), presumably reflecting the basal permeability of the plasma membrane to Ba^{2+}. After exposure of the cells to ionomycin (1 μM for 5 min), the rate of fluorescence increase due to Ba^{2+} addition was $(3{\cdot}1 \pm 0{\cdot}7)$-fold greater than the basal rate ($n = 4$), suggesting that the capacitative pathway had been activated and that Ba^{2+} permeated it. The rate of Ba^{2+} entry was further increased by addition of AVP (10 nM) (Fig. 5*A* and *B*). The rate of fluorescence increase slowed immediately after removal of extracellular Ba^{2+} (Fig. 5*C*), although the fluorescence signal remained elevated, probably because Ba^{2+} is a poor substrate for Ca^{2+}-ATPases and is not, therefore, effectively removed from the cytosol (Palade, Dettbarn, Brunder, Stein & Hals, 1989).

The increased rates of bivalent cation entry after addition of AVP are not merely a consequence of an AVP-evoked change in membrane potential because substantial replacement of extracellular Na^+ with K^+ (extracellular NaCl concentration ($[NaCl]_o$) = 5·9 mM, extracellular KCl concentration ($[KCl]_o$) = 135 mM; Fig. 5*D*) or complete replacement with the impermeant cation NMDG (not shown) did not prevent AVP from stimulating Ba^{2+} entry. Further evidence that the effect of AVP results from activation of a pathway permeable to bivalent cations, rather than an increase in the electrochemical gradient for their entry, is provided by our observation that under similar conditions AVP does not stimulate Mn^{2+} entry (see below). The ability of AVP to stimulate Ba^{2+} and Sr^{2+} entry in cells pretreated with ionomycin (Figs 4 and 5) therefore suggests that AVP can stimulate a second entry pathway additional to that activated by empty stores. To eliminate the possibility that AVP is simply emptying the stores more completely than ionomycin alone, cells were treated with both ionomycin (1 μM) and thapsigargin (500 nM) in Ca^{2+}-free medium; this treatment should both fully empty the stores and prevent their refilling. After this pretreatment, the cells were exposed to extracellular Ba^{2+} (1·5–3 mM), which resulted in a steady rate of increase in the fura-2 fluorescence ($\lambda_{ex} = 360$ nm); subsequent addition of AVP (20 nM) further increased the rate of Ba^{2+} entry (by

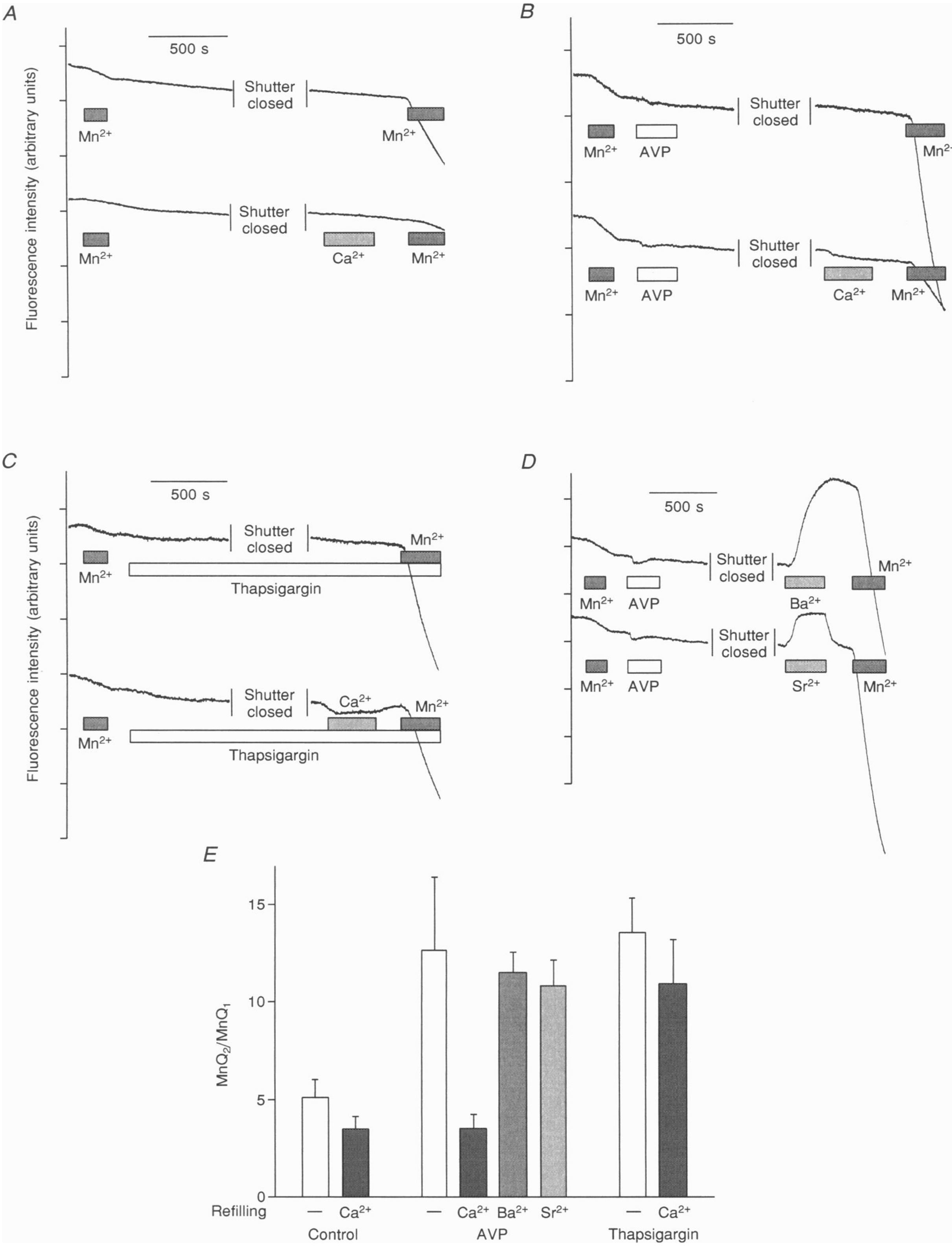

Figure 3. The capacitative entry pathway monitored by Mn^{2+} quenching of fura-2

A–D, Mn^{2+} quenching of the fura-2 fluorescence ($\lambda_{ex} = 360$ nm) of cell populations was recorded in Ca^{2+}-free medium during brief exposures (150–250 s) to $MnCl_2$ (0·5 mM, dark grey bars). The first exposure (150 s) established the basal rate of quench (MnQ_1), and the second exposure (250 s) allowed the effects of various treatments to be measured (MnQ_2); the shutter was closed, protecting the cells from the light source, for 500 s between the two perfusions with $MnCl_2$. Cells were exposed for the times shown to AVP (50 nM for 250 s; *B* and *D*) or thapsigargin (500 nM for 1650 s; *C*) to empty their intracellular Ca^{2+} stores.

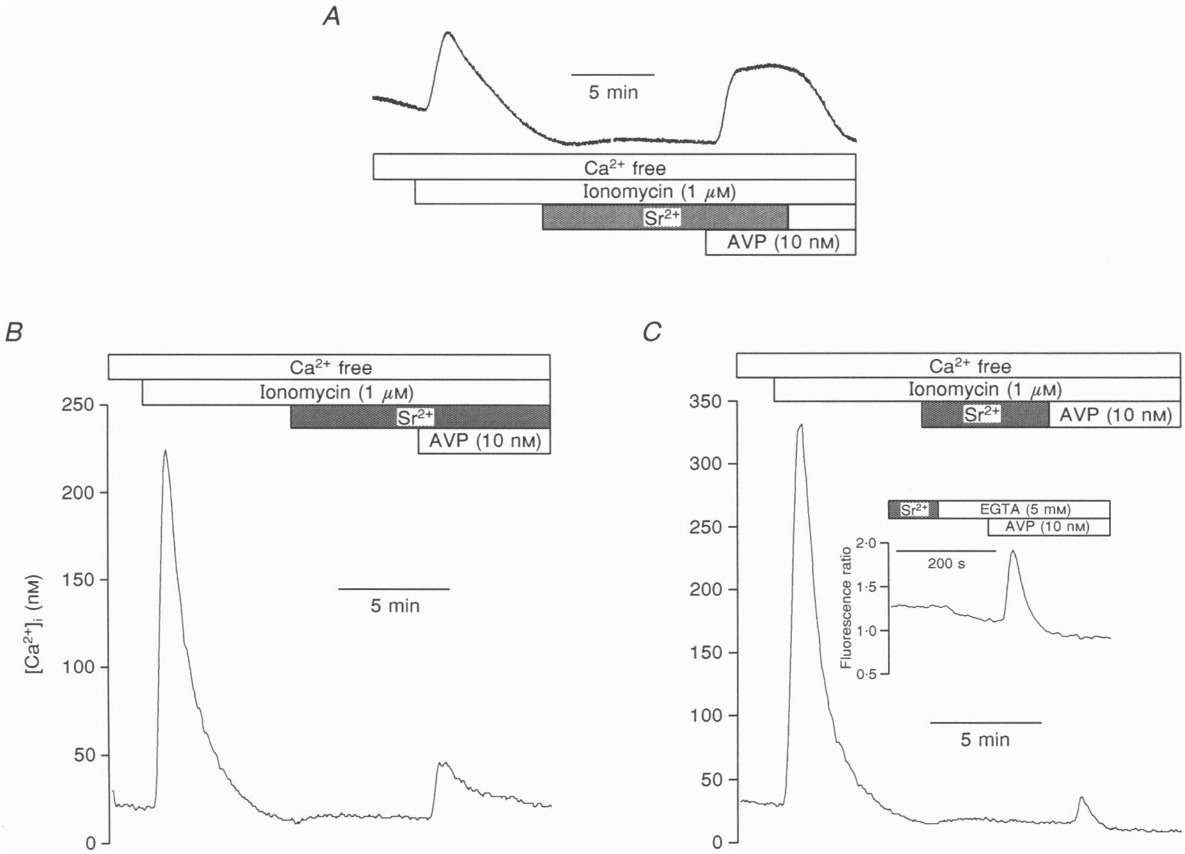

Figure 4. Effects of AVP on Sr^{2+} entry

A, single wavelength recording ($\lambda_{ex} = 340$ nm) from a population of cells treated as indicated. All media were Ca^{2+} free (+ 0·1 mM EGTA) and contained nimodipine (50 nM). $SrCl_2$ (2 mM + 0·5 mM EGTA) was included in the medium for the time shown by the grey bar. *B* and *C*, calibrated recordings from cell populations excited alternately with 340 and 380 nm light; the calibration refers to $[Ca^{2+}]_i$ and not to the Sr^{2+} signal. All media were Ca^{2+} free (+ 0·1 mM EGTA) and contained nimodipine (50 nM). $SrCl_2$ (2·5 mM + 1 mM EGTA) was included in the medium for the time shown by the grey bar. In *C*, EGTA (5 mM) was added with the AVP (10 nM) to ensure complete chelation of the extracellular Sr^{2+}. The inset shows results for a similar experiment in which AVP (10 nM) was added 100 s after perfusion of the cells with Sr^{2+}-free medium containing 5 mM EGTA, confirming that the transient increase in fluorescence ratio is not due to influx of extracellular Sr^{2+}. Results typical of at least 3 similar experiments are shown in each of the panels. Similar results were observed in measurements from single cells (not shown).

The lower traces of *A–C* illustrate the effects of adding $CaCl_2$ to the medium (10 mM for 5 min) before the second addition of $MnCl_2$, and *D* illustrates the effects of adding $BaCl_2$ (10 mM for 5 min, upper trace) or $SrCl_2$ (10 mM for 5 min, lower trace) prior to the second measurement of Mn^{2+} entry. The increases in fluorescence during exposure to extracellular Ba^{2+} or Sr^{2+} result from the binding of these ions to fura-2, the fluorescence of which is sensitive to these ions at the excitation wavelength used (360 nm). Experiments performed using the isosbestic wavelengths at which fura-2 fluorescence is insensitive to Ba^{2+} or Sr^{2+} ($\lambda_{ex} = 370$ and 364 nm, respectively; Byron & Taylor, 1993) produced similar Mn^{2+} quench results (not shown). All solutions contained verapamil (10 μM) and 0·1 mM EGTA. *E*, results from 3–5 experiments similar to those shown in *A–D* are summarized. Quench rates of fura-2 fluorescence during the first and second exposures to $MnCl_2$ (MnQ_1 and MnQ_2, respectively) were determined by fitting a straight line by least-squares regression to the fluorescence trace for the 50 s period beginning 100 s after addition of $MnCl_2$; all rates were corrected for non-specific loss of signal by subtracting the rate of fluorescence decrease during the 100 s prior to closing the shutter. Mean ratios of these corrected rates (MnQ_2/MnQ_1) are shown for each of the conditions shown in *A–D*; error bars are S.E.M.

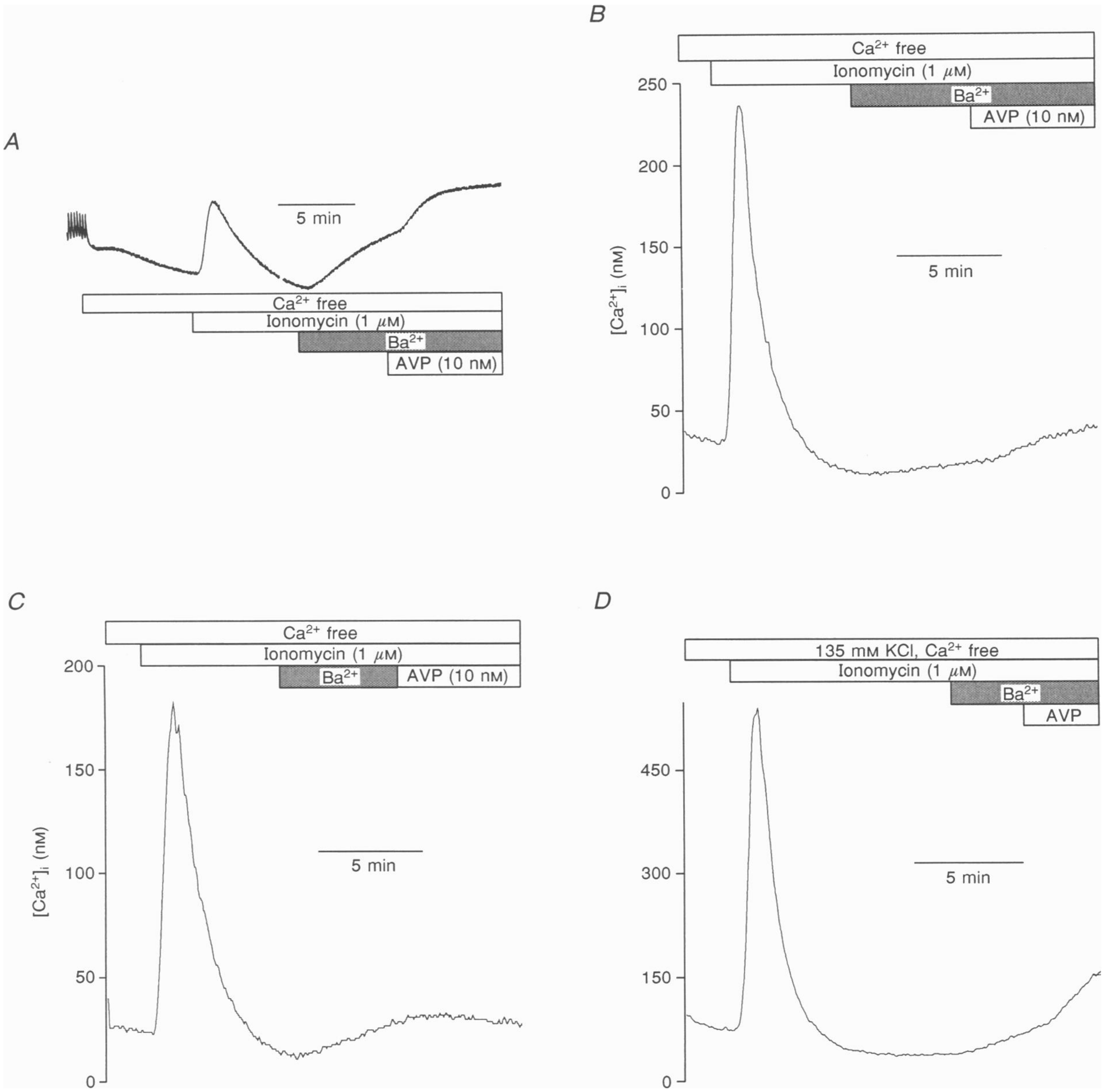

Figure 5. Effects of AVP on Ba^{2+} entry

A, single wavelength recording (λ_{ex} = 340 nm) from a population of cells treated as indicated. Prior to addition of nimodipine (50 nM), cells displayed spontaneous Ca^{2+} spiking (Byron & Taylor, 1993); all subsequent perfusions were with Ca^{2+}-free media (+ 0·1 mM EGTA) containing nimodipine (50 nM). $BaCl_2$ (1·5 mM + 0·1 mM EGTA) was included in the extracellular medium for the time shown by the grey bar. Similar results were obtained from experiments in which λ_{ex} was 360 nm, confirming that the increase in fura-2 fluorescence reflects an increase in [Ba^{2+}] and not an increase in [Ca^{2+}] (see text for further details). *B* and *C*, calibrated recordings from cell populations excited alternately with 340 and 380 nm light; the calibration refers to $[Ca^{2+}]_i$ and not to the Ba^{2+} signal. All media were Ca^{2+} free (+ 0·1 mM EGTA) and contained nimodipine (50 nM). $BaCl_2$ (1·5 mM + 1 mM EGTA) was included in the extracellular media for the times shown by the grey bars. In *C*, EGTA (5 mM) was added with the AVP (10 nM) to ensure complete chelation of the extracellular Ba^{2+}. *D*, calibrated recordings from cell populations excited alternately with 340 and 380 nm light; the calibration refers to $[Ca^{2+}]_i$ and not to the Ba^{2+} signal. All media were Ca^{2+} free (+ 0·1 mM EGTA) and contained verapamil (10 μM). The cells were perfused with a depolarizing medium ($[KCl]_o$ = 135 mM, $[NaCl]_o$ = 5·9 mM) before addition of ionomycin (1 μM), $BaCl_2$ (3 mM + 1 mM EGTA; grey bar) and AVP (10 nM). Results typical of at least 3 similar experiments are shown in each of the panels. Similar results to those shown in *B* and *C* were observed in measurements from single cells (not shown).

2·95-fold, $n = 2$; Fig. 6A). These experiments were repeated using single cell image analysis with similar results (Fig. 6B), confirming that the results from populations reflect the behaviour of the entire cell population and not the behaviour of subpopulations of cells with different properties.

The results shown in Fig. 2 demonstrate that the capacitative entry pathway is permeable to Mn^{2+}. The protocol shown in Fig. 7A was used to test whether the second bivalent cation entry pathway is also permeable to Mn^{2+}. Cells were pretreated with thapsigargin (500 nM for 30 min) in Ca^{2+}-free medium to empty their intracellular Ca^{2+} stores and maximally activate capacitative Mn^{2+} entry. The cells were then exposed first to medium containing 30 μM $MnCl_2$ for 150 s, then to medium containing 90 μM $MnCl_2$ for 150 s, and finally returned to medium containing 30 μM $MnCl_2$. The rates of fura-2 quenching were measured during the last 50 s of each of these incubations. The results demonstrate that the rate of Mn^{2+} entry reversibly increased (by $5·8 \pm 1·7$-fold, $n = 4$) when the extracellular Mn^{2+} concentration was increased from 30 to 90 μM (Fig. 7B), indicating that the lower concentration of extracellular Mn^{2+} (30 μM) saturated neither the capacitative entry pathway nor our ability to detect its activity.

Addition of AVP (20 nM) during the second exposure to 30 μM $MnCl_2$, when a stable rate of fluorescence quench had been attained, had no effect on the fura-2 quench rate, although again increasing the extracellular $MnCl_2$ concentration to 90 μM reversibly increased the rate of fluorescence quenching by $3·8 \pm 1·2$-fold ($n = 3$). These results demonstrate that under conditions that would allow a further increase in Mn^{2+} entry to be detected, AVP neither increased Mn^{2+} entry beyond that already activated by store depletion, nor inhibited capacitative Mn^{2+} entry. The second AVP-stimulated bivalent cation entry pathway is probably not, therefore, significantly permeable to Mn^{2+}.

AVP activates Ca^{2+} extrusion

During prolonged stimulation with AVP (50 nM), $[Ca^{2+}]_i$ falls to below the original baseline (Fig. 1) suggesting that Ca^{2+} entry is more than compensated by increased Ca^{2+} extrusion or reuptake. This effect was further investigated by examining the effects of AVP on cells in which $[Ca^{2+}]_i$ had been elevated by incubation with thapsigargin (500 nM) in Ca^{2+}-containing medium. Addition of AVP (50 nM) to cells in which $[Ca^{2+}]_i$ had been increased to about 400 nM, caused a rapid (within 60 s) decrease in $[Ca^{2+}]_i$, followed by a slow recovery over several minutes towards the initial elevated $[Ca^{2+}]_i$ (Fig. 8A). Similar results were obtained from single cells (Fig. 8B). Since the activities of intracellular Ca^{2+}-ATPases were inhibited by thapsigargin in these experiments and AVP does not inhibit the activity of the capacitative entry pathway (Fig. 7), the ability of AVP to reduce $[Ca^{2+}]_i$ probably results from activation of a Ca^{2+} efflux pathway.

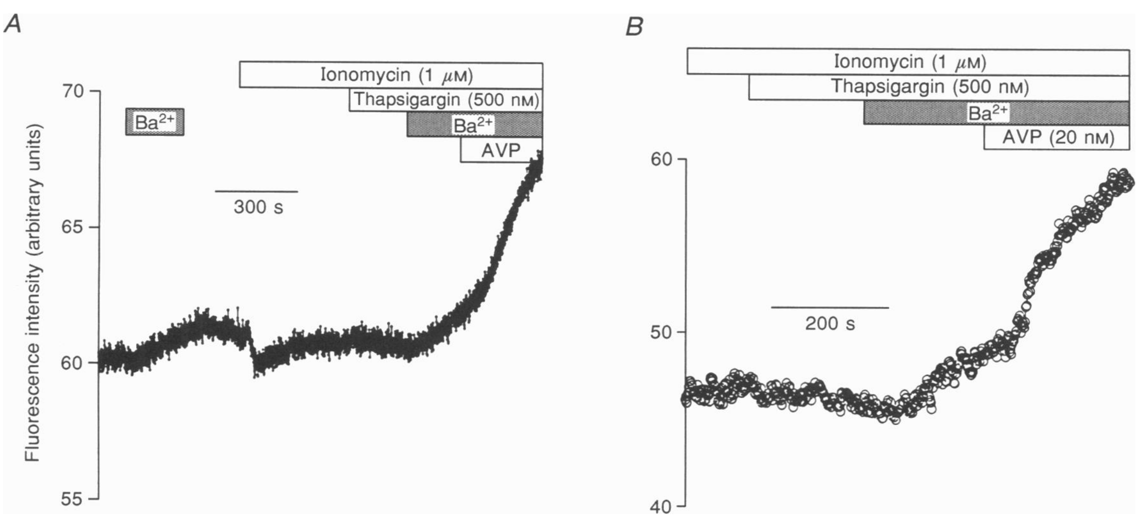

Figure 6. AVP enhances Ba^{2+} entry in the presence of thapsigargin and ionomycin

A, single wavelength recording ($\lambda_{ex} = 360$ nm) from a population of cells treated as indicated. All media were Ca^{2+} free (+ 0·1 mM EGTA) and contained verapamil (10 μM). $BaCl_2$ (2 mM, grey bar) was included in the extracellular medium for the times shown. [AVP], 20 nM. Results are representative of 4 similar experiments. B, single wavelength recording ($\lambda_{ex} = 360$ nm) from a single cell pretreated for 5 min with ionomycin (1 μM), then treated as indicated. All media were Ca^{2+} free (+ 0·1 mM EGTA) and contained verapamil (10 μM). $BaCl_2$ (2 mM, grey bar) was included in the extracellular medium for the time shown. Results are representative of 3 similar experiments.

DISCUSSION

In A7r5 cells, as in many other cell types (Putney, 1990), depletion of intracellular Ca^{2+} stores with thapsigargin evoked a sustained increase in $[Ca^{2+}]_i$ (Fig. 2) reflecting activation of a capacitative Ca^{2+} entry pathway. This capacitative pathway was also permeable to Mn^{2+} (Figs 3 and 7), Ba^{2+} (Figs 5 and 6) and probably Sr^{2+}, although Sr^{2+} entry was detectable only when the extracellular Sr^{2+} concentration ($[Sr^{2+}]_o$) was substantially increased (to 10 mM, Fig. 3*D*; compare Figs 2, 4*B* and 4*C* where $[Sr^{2+}]_o = 1{\cdot}5$ mM). These results are consistent with electrophysiological measurements of the current passing through the capacitative pathway (I_{CRAC}, Ca^{2+} release-activated Ca^{2+} current) of mast cells (Hoth & Penner, 1992, 1993), *Xenopus* oocytes (Parekh, Terlau & Stühmer, 1993) and T lymphocytes (Zweifach & Lewis, 1993), which suggests that I_{CRAC} is unusual among Ca^{2+} channels in being more permeable to Ca^{2+} than to Ba^{2+}, Sr^{2+} or Mn^{2+}, although each of these cations permeates to some degree.

Refilling of the intracellular stores with Ca^{2+} reversed the activation of the capacitative Ca^{2+} entry pathway (Fig. 3*A–C*); however, neither Sr^{2+} nor Ba^{2+} were effective (Fig. 3*D* and *E*). Sr^{2+} was accumulated by the intracellular stores (Fig. 4*C*), suggesting that its lack of effect on the capacitative pathway results from its inability to mimic the effects of luminal Ca^{2+}. In contrast to the results with Sr^{2+} (Fig. 4*C*), prolonged stimulation of Ba^{2+} entry followed by addition of AVP (10 nM) with the concomitant removal of extracellular Ba^{2+} had no effect on fura-2 fluorescence (Fig. 5*C*). Since Ba^{2+} is known to permeate InsP_3 receptors (Bezprozvanny & Ehrlich, 1993), these results suggest that Ba^{2+} is not accumulated within intracellular stores. The inability of Ba^{2+} to inactivate the capacitative pathway is not, therefore, surprising. In platelets too, preincubation with Ba^{2+} failed to inactivate capacitative Mn^{2+} entry, although refilling of stores with Sr^{2+} has been reported to inactivate the pathway (Ozaki, Yatomi & Kume, 1992; Jenner, Farndale & Sage, 1994). While our work was in progress, another report of AVP effects on bivalent cation fluxes in A7r5 cells appeared (Hughes & Schachter, 1994). The conclusions of that work conflict with many previous reports (Galizzi, Qar, Fosset, Van Renterghem & Lazdunski, 1987; Van Renterghem, Romey & Lazdunski, 1988; Missiaen *et al.* 1990; Giannattasio, Jones & Scarpa, 1991; Otun, Gillespie, Nicholls, Greenwell & Dunlop, 1992; Byron & Taylor, 1993) and with the conclusions of the present study, notably in suggesting that neither Ba^{2+} nor Mn^{2+}

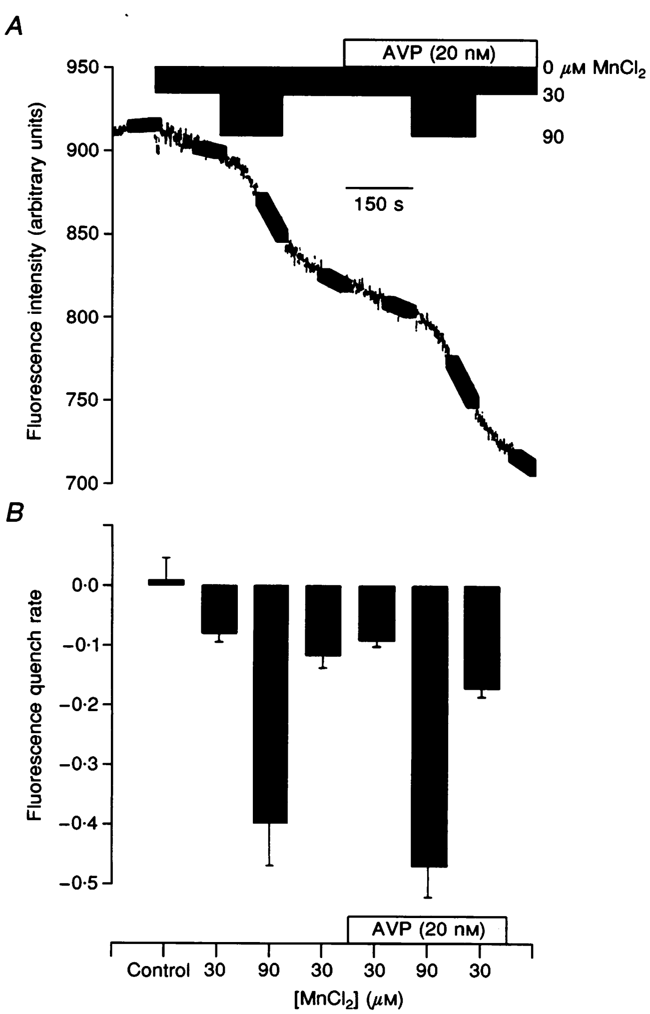

Figure 7. AVP does not stimulate further Mn^{2+} entry to cells with empty Ca^{2+} stores

A, single wavelength recording ($\lambda_{ex} = 360$ nm) from a population of cells pretreated with thapsigargin (500 nM) in Ca^{2+}-free medium for 30 min to empty their intracellular Ca^{2+} stores and maximally activate the capacitative entry pathway. All media were nominally Ca^{2+} free (EGTA was omitted because it binds Mn^{2+} with greater affinity than Ca^{2+}) and contained thapsigargin (500 nM) and verapamil (10 µM). $MnCl_2$, at 30 and 90 µM, was included in the medium for the periods shown. AVP (20 nM) was included for the second half of the incubation as shown by the open bar. The filled bars superimposed on the traces show the straight lines fitted by least-squares regression to the final 50 s of each 150 s treatment (see Results). *B*, results from 4 independent experiments similar to those shown in *A* are summarized. Each fluorescence quench rate (mean ± S.E.M.) is plotted as the slope of the line fitted by least-squares linear regression to the final 50 s of each exposure to $MnCl_2$.

Mn^{2+} permeates the capacitative pathway. The latter discrepancy probably results from the near complete activation of the capacitative pathway prior to the attempts of Hughes & Schachter (1994) to activate it experimentally, a conclusion supported by the minimal effects of thapsigargin and ionomycin on Ca^{2+} mobilization, the very large 'basal' Mn^{2+}, Ba^{2+} and Ca^{2+} influxes, and the near complete inhibition of the latter by Ni^{2+}.

In many cells, the capacitative Ca^{2+} entry pathway appears to provide a sufficient explanation for the Ca^{2+} entry evoked by activation of receptors linked to InsP_3 formation (Takemura, Hughes, Thastrup & Putney, 1989; Jacob, 1990; Chow & Jondal, 1990; Zweifach & Lewis, 1993; Demaurex, Monod, Lew & Krause, 1994). However, recent results from several studies have been interpreted as evidence in favour of a second, receptor-regulated Ca^{2+} entry pathway, additional to that activated by empty stores, but the methods used have not always eliminated the possibility that receptor activation is merely more effectively emptying the intracellular stores, and so more completely activating the capacitative pathway (Stauderman & Pruss, 1989; Hansen, Yang & Williamson, 1991). This is a difficult issue to resolve because a fraction of the intracellular Ca^{2+} stores, perhaps those immediately beneath the plasma membrane, may be largely responsible for regulation of the capacitative pathway and yet contribute relatively little to Ca^{2+} mobilization. An additional problem is that in some cells, notably hepatocytes, fura-2 trapped within intracellular stores may be quenched by Mn^{2+} only when InsP_3 receptors are activated (Glennon, Bird, Kwan & Putney, 1992); an enhanced rate of Mn^{2+} quench of fura-2 fluorescence after receptor activation could then be mistakenly interpreted as activation of an additional entry pathway (Kass, Llopis, Chow, Duddy & Orrenius, 1990, but see Kass, Webb, Chow, Llopis & Berggren, 1994).

In examining the Ca^{2+} entry pathways in A7r5 cells, we have designed experiments that overcome these pitfalls. Firstly, intracellular compartmentalization of fura-2 does not appear to be a significant problem in A7r5 cells: 95% of the indicator is rapidly released from the cells when they are permeabilized with saponin (see Methods; Byron & Taylor, 1993), and there is no further quench of fura-2 fluorescence when AVP is added after the cytosolic fura-2 has been fully quenched by incubation with thapsigargin and extracellular $MnCl_2$ (not shown). Secondly, we have verified that the effects of AVP on the second entry pathway cannot be attributed to more substantial activation of the capacitative pathway (see below). Thirdly, by combining studies of single cells and cell populations, we have verified that activation of an additional pathway does not reflect recruitment of cells; this is important because we have observed considerable heterogeneity in the effects of thapsigargin on single cells. Finally, by demonstrating that AVP stimulates Ba^{2+} entry when extracellular Na^+ is replaced by NMDG or K^+ (Fig. 5*D*), and that the pathway is selective for specific bivalent cations (see below), we have confirmed that the effects of AVP reflect stimulation of a bivalent cation entry pathway rather than stimulation of Na^+–Ca^{2+} exchange or an increase in the electrochemical gradient favouring bivalent cation influx.

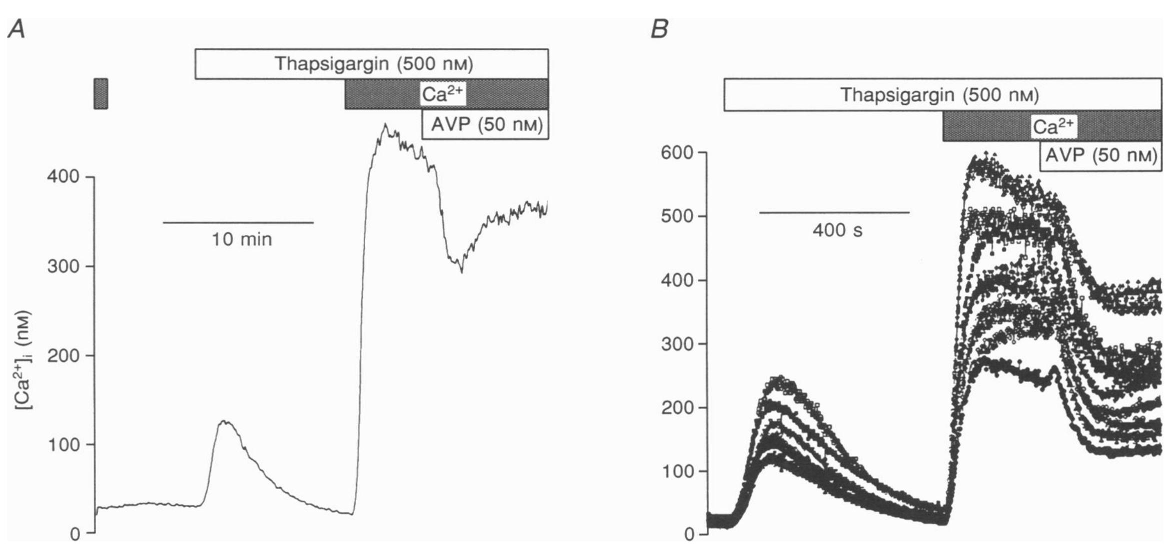

Figure 8. AVP activates Ca^{2+} extrusion from thapsigargin-treated cells

A, calibrated recording, typical of 5 similar experiments, from a population of cells treated as indicated. *B*, superimposed recordings from 8 individual cells in the same microscopic field treated as indicated. Results are typical of 2 similar experiments from different coverslips. All media in both panels contained verapamil (10 μM) and were Ca^{2+} free (+ 0·1 mM EGTA) except where indicated by the grey bars (where $CaCl_2$ = 1·5 mM).

The results shown in Figs 4–6 demonstrate the presence in A7r5 cells of a second bivalent cation entry pathway that is activated by AVP and conducts Ba^{2+} and Sr^{2+}, but not Mn^{2+} (Fig. 7). In the experiments shown in Figs 4 and 5, we used ionomycin to deplete intracellular Ca^{2+} stores and thereby activate the capacitative pathway; extracellular Ba^{2+} or Sr^{2+} were then added before addition of AVP. Since ionomycin does not transport Ba^{2+} or Sr^{2+}, it is important to consider the possibility that the stores might refill with Ba^{2+} or Sr^{2+}; the subsequent stimulation of bivalent cation entry by AVP might then simply reflect re-emptying of the stores and consequent activation of capacitative Sr^{2+} or Ba^{2+} entry. This cannot be the explanation for several reasons. (1) With a similar extracellular concentration of Sr^{2+} (1·5 mM) the capacitative pathway, when activated by treatment with thapsigargin, did not cause detectable Sr^{2+} entry (Fig. 2). (2) Neither Ba^{2+} nor Sr^{2+} appeared to be capable, under these conditions, of inactivating the capacitative pathway (Fig. 3*D* and *E*). (3) AVP stimulated Ba^{2+} entry into cells in which stores had been emptied by ionomycin and prevented from refilling by co-incubation with thapsigargin (Fig. 6). (4) Under conditions in which AVP stimulated both Ba^{2+} and Sr^{2+} entry (Figs 4 and 5), it did not stimulate Mn^{2+} entry (Fig. 7). Since the capacitative entry pathway was permeant to Mn^{2+} (Fig. 3), this result confirms that AVP cannot be acting by further activation of the capacitative pathway. We conclude that AVP, in addition to its ability to activate capacitative Ca^{2+} entry by emptying InsP_3-sensitive Ca^{2+} stores, also activates an additional bivalent cation entry pathway.

Although our studies of ion permeation have succeeded in distinguishing capacitative and AVP-regulated bivalent cation entry pathways in A7r5 cells, the rise in $[Ca^{2+}]_i$ resulting from both thapsigargin- and AVP-stimulated Ca^{2+} entry were similarly inhibited by other bivalent cations (not shown) and with the same relative effectiveness as reported for I_{CRAC} by Penner, Fasolato & Hoth (1993) ($Be^{2+} > Zn^{2+} > Ni^{2+} > Sr^{2+}$). Our results do not, however, distinguish between two possibilities: either both entry pathways have similar sensitivities to blockade in A7r5 cells, or the capacitative pathway is the major component of AVP-stimulated Ca^{2+} entry detected by fura-2.

Our results are consistent with another study of A7r5 cells in which the general anaesthetics halothane and isoflurane were shown to substantially attenuate AVP-stimulated Ca^{2+} mobilization and to have far lesser effects on AVP-stimulated Ca^{2+} entry (Sill, Eskuri, Nelson, Tarar & Van Dyke, 1993). Both effects of AVP are likely to be mediated by a single class of receptor, since radioligand binding studies have convincingly demonstrated the presence of only the V_1-subclass of AVP receptor (Thibonnier, Bayer, Simonson & Kester, 1991), and we have shown that a selective peptide antagonist of the V_{1a}-vasopressin receptor abolishes the effects of AVP on both Ca^{2+} mobilization and Sr^{2+} entry.

The nature of the signal that regulates the second bivalent cation entry pathway in A7r5 cells is unknown. It is unlikely that the vasopressin receptor is itself a Ca^{2+} channel because the only high affinity vasopressin binding sites in A7r5 cells have the pharmacology typical of V_1-vasopressin receptors (Thibonnier *et al.* 1991), and these receptors are structurally unrelated to known ion channels. InsP_4, which stimulates Ca^{2+} entry in some cells (Irvine & Cullen, 1993), is a possible candidate, but electrophysiological recordings from endothelial cells suggest that the InsP_4-activated channel is equally permeable to Ca^{2+}, Ba^{2+} and Mn^{2+} (Lückhoff & Clapham, 1992). Another possible regulator of the second entry pathway is InsP_3 acting directly at a plasma membrane receptor (Khan, Steiner, Klein, Schneider & Snyder, 1992), but neither this mechanism nor an involvement of InsP_4 can be readily reconciled with the ability of anaesthetics to partially dissociate AVP-stimulated Ca^{2+} mobilization from Ca^{2+} entry in A7r5 cells (Sill *et al.* 1993). Regulation by protein kinase C (Oike, Kitamura & Kuriyama, 1993) or perhaps direct regulation of an entry pathway by a G protein could also explain the ability of AVP to activate the second entry pathway.

AVP appears to bind to only a single class of receptor, the V_1-vasopressin receptor, in A7r5 cells (Thibonnier *et al.* 1991), yet it regulates the activities of many Ca^{2+} transport pathways. AVP, presumably via its ability to stimulate InsP_3 formation, causes mobilization of intracellular Ca^{2+} stores and consequent activation of capacitative Ca^{2+} entry. In addition, AVP activates a second distinct bivalent cation entry pathway and a Ca^{2+} efflux pathway; the signals linking V_1-vasopressin receptors to these Ca^{2+} transport pathways are unknown. These multiple effects of AVP, which are modulated by AVP concentration, determine both the nature and the amplitude of the $[Ca^{2+}]_i$ signal.

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