# Receptors linked to polyphosphoinositide hydrolysis stimulate $Ca^{2+}$ extrusion by a phospholipase C-independent mechanism

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In A7r5 cells with empty intracellular $Ca^{2+}$ stores in which the cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) had been increased by capacitative $Ca^{2+}$ entry, stimulation of receptors linked to phospholipase C (PLC), including those for $Arg^8$-vasopressin (AVP) and platelet-derived growth factor (PDGF), caused a decrease in $[Ca^{2+}]_i$. This effect was further examined in a stable variant of the A7r5 cell line in which the usual ability of hormones to stimulate non-capacitative $Ca^{2+}$ entry is not expresssed. In thapsigargin-treated cells, neither AVP nor PDGF affected capacitative $Mn^{2+}$ or $Ba^{2+}$ entry, but both stimulated the rate of $Ca^{2+}$ extrusion, and their abilities to decrease $[Ca^{2+}]_i$ were only partially inhibited by removal of extracellular $Na^+$. These results suggest that receptors linked to PLC also stimulate plasma membrane $Ca^{2+}$ pumps. Activation of protein kinase C by phorbol 12,13-dibutyrate (PDBu, 1 $\mu$M) also caused a decrease in $[Ca^{2+}]_i$ by accelerating $Ca^{2+}$ removal from the cytosol; the effect was again only partially inhibited by removal of extracellular $Na^+$. An inhibitor of PKC, Ro31-8220 (10 $\mu$M), abolished the ability of PDBu to decrease $[Ca^{2+}]_i$,without affecting the response to maximal or submaximal concentrations of AVP. Similar experiments with PDGF were impracticable because Ro31-8220, presumably by inhibiting the tyrosine kinase activity of the PDGF receptor, abolished all responses to PDGF. U73122 (10 $\mu$M), an inhibitor of PLC, completely inhibited PDGF- or AVP-evoked $Ca^{2+}$ mobilization, without preventing either stimulus from causing a decrease in $[Ca^{2+}]_i$. We conclude that receptors coupled to PLC, whether via G-proteins or protein tyrosine kinase activity, also share an ability to stimulate the plasma membrane $Ca^{2+}$ pump via a mechanism that does not require PLC activity.

Key words: $Ca^{2+}$ pump, platelet-derived growth factor, protein kinase C, vasopressin.

## INTRODUCTION

The ability of many hormones, growth factors and neurotransmitters to stimulate the opening of $Ca^{2+}$ channels in either the membranes of intracellular $Ca^{2+}$ stores or the plasma membrane is largely responsible for the rapid increases in cytosolic free $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) evoked by such stimuli. For many cells, the most important means of regulating these channels is provided by receptor-stimulated polyphosphoinositide hydrolysis [1]. Inositol 1,4,5-trisphosphate ($Ins P_3$) and the intrinsic $Ca^{2+}$ channel of the receptors for $Ins P_3$ [2] provide the link between receptors in the plasma membrane and release of intracellular $Ca^{2+}$ stores. $Ca^{2+}$ entry across the plasma membrane often involves $Ca^{2+}$ channels that are stimulated by depletion of the intracellular $Ca^{2+}$ stores, the capacitative $Ca^{2+}$ entry pathway [3]. According to cell type, additional non-capacitative $Ca^{2+}$ entry pathways may also be involved [4,5]. Capacitative $Ca^{2+}$ entry is likely to be mediated by assemblies of proteins related to the *Drosophila* trp and trpl proteins [6,7], whereas non-capacitative pathways are a heterogenous collection of channels that include many non-selective cation channels [4,8–11], some of which may also be assembled from trp and trpl proteins.

Increases in $[Ca^{2+}]_i$ are potentially dangerous [12,13]. Both the rapid passive fluxes of $Ca^{2+}$ through open channels and the active extrusion of $Ca^{2+}$ from the cytosol are therefore subject to rigorous control. Cytosolic $Ca^{2+}$ itself is probably the most widespread means of regulating these $Ca^{2+}$ transport processes. Most, if not all, $Ca^{2+}$ channels are subject to feedback inhibition by increases in $[Ca^{2+}]_i$, a characteristic that is certainly a feature of both $Ins P_3$ receptors [14] and the capacitative $Ca^{2+}$ entry pathway [15]. The proteins responsible for removing $Ca^{2+}$ from the cytosol, notably the plasma membrane $Ca^{2+}$ pump [16–18], are stimulated by cytosolic $Ca^{2+}$ and so provide a further defence against excessive increases in $[Ca^{2+}]_i$. In addition to these important autoregulatory effects of increased $[Ca^{2+}]_i$ on $Ca^{2+}$ transport, the same stimuli that cause $Ca^{2+}$ channels to open may also stimulate $Ca^{2+}$ removal from the cytosol, independent of their abilities to increase $[Ca^{2+}]_i$ [19–24].

A7r5 cells are a vascular smooth muscle cell line [25] in which we have established that $Arg^8$-vasopressin (AVP) regulates several different $Ca^{2+}$ transport processes: release of intracellular $Ca^{2+}$ stores mediated by $Ins P_3$ and consequent activation of capacitative $Ca^{2+}$ entry [26]; activation, via arachidonic acid, of a non-capacitative $Ca^{2+}$ entry pathway [4]; and activation of a process that leads to a decrease in $[Ca^{2+}]_i$ [26]. In the present study, we focus on the ability of agonists to stimulate recovery from an increase in $[Ca^{2+}]_i$ and conclude that whereas activation of protein kinase C (PKC) mimics the effects of receptor activation, neither PKC nor phospholipase C (PLC) is involved in the ability of receptors with intrinsic tyrosine kinase activity or those that couple to G-proteins to stimulate $Ca^{2+}$ extrusion.

## MATERIALS AND METHODS

### Cell culture and $[Ca^{2+}]_i$ measurements

The methods have been described in detail previously [4,26]. Briefly, A7r5 cells were grown in Dulbecco's modified Eagle's

Abbreviations used: AVP, $Arg^8$-vasopressin; 5-HT, 5-hydroxytryptamine; PDBu, phorbol 12,13-dibutyrate; PDGF, platelet-derived growth factor; PKC, protein kinase C; $[Ca^{2+}]_i$, cytosolic free $Ca^{2+}$ concentration; fura-2/AM, fura-2 acetoxymethyl ester; HBS, Hepes-buffered saline; PLC, phospholipase C; SERCA, sarcoplasmic/endoplasmic-reticulum $Ca^{2+}$-ATPase; $t_{1/2}$, half-time for recovery of $[Ca^{2+}]_i$.

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medium supplemented with 10 % foetal calf serum, L-glutamine (3.8 mM) and 0.9 % non-essential amino acids [26]. To simplify analysis of the $Ca^{2+}$ removal pathways, all the experiments reported herein used an A7r5 cell line in which hormones are unable to stimulate the non-capacitative $Ca^{2+}$ entry pathway [4] (see the Results and discussion section). In this cell line, which was serendipitously established in a previous study, the regulation of $Ca^{2+}$ signalling by AVP and other agonists is indistinguishable from the parental cells, except for the inability of hormones to stimulate the non-capacitative pathway [4]. For measurements of $[Ca^{2+}]_i$, cells were subcultured onto rectangular glass coverslips (No. 2, $9 \times 22$ mm) and used 1–5 days later when they were confluent. All measurements were made in Hepes-buffered saline (HBS: NaCl 135 mM, KCl 5.9 mM, $MgCl_2$ 1.2 mM, $CaCl_2$ 1.5 mM, Hepes 11.6 mM, glucose 11.5 mM, pH 7.3). Cells were loaded with fura-2 by incubation in HBS containing 2 $\mu$M fura-2 acetoxymethyl ester (fura-2/AM), 0.02 % pluronic acid F-127 and 1 mg/ml BSA for 120 min, followed by a further 60 min in HBS containing 1 mg/ml BSA. Fura 2-loaded cells were mounted in a cuvette and placed in a Hitachi F-4500 spectrofluorimeter and fluorescence was measured at 1 s intervals (excitation wavelengths = 340, 359 and 380 nm; emission wavelength = 510 nm). Autofluorescence was determined at the end of each experiment by addition of ionomycin (1 $\mu$M) and $MnCl_2$ (1 mM) in $Ca^{2+}$-free HBS and the corrected fluorescence ratios were calibrated to $[Ca^{2+}]_i$ by reference to a look-up table [26]. Rates of recovery from elevated $[Ca^{2+}]_i$ in cells with empty stores were determined by rapid exchange (complete in $< 3$ s) of normal HBS for $Ca^{2+}$-free HBS supplemented with 100 $\mu$M EGTA. Rates of $Mn^{2+}$ and $Ba^{2+}$ entry were measured in HBS in which $CaCl_2$ was replaced by 10–100 $\mu$M $Mn^{2+}$ or 2 mM $BaCl_2$. The temperature in all experiments was 22 °C and all media included 200 nM nimodipine to inhibit L-type $Ca^{2+}$ channels [27].

Results are expressed as means $\pm$ S.E.M. and significance was assessed using paired or unpaired Student's *t*-test as appropriate with $P < 0.05$ considered significant.

## Materials

Cell culture media were from Gibco BRL (Paisley, U.K.). Nimodipine was from Tocris Cookson (Bristol, U.K.). Foetal calf serum was from Advanced Protein Products (Brierley Hill, U.K.). Fura 2-AM and pluronic F-127 were from Molecular Probes (Leiden, The Netherlands). Ionomycin, Hepes, U73122 and human recombinant platelet-derived growth factor-BB (PDGF-BB) were from Calbiochem (Nottingham, U.K.). Ro31-8220 and RHC 80267 were from Alexis Corporation Ltd (Nottingham, U.K.). Thapsigargin was from Alamone Laboratories (Jerusalem, Israel). LOE-908 was a gift from Boehringer Ingeheim. Bis-phenol [bis(2-hydroxyl-3-t-butyl-5-methyl phenyl) methane] from Pfaltz and Bauer (Flushing, NY, U.S.A.) was a gift from Frank Michelangeli (University of Birmingham, Birmingham, U.K.). All other reagents, including AVP, phorbol 12,13-dibutyrate (PDBu) and bombesin were from Sigma.

## RESULTS AND DISCUSSION

### $Ca^{2+}$-mobilizing stimuli cause a decrease in the $[Ca^{2+}]_i$ of A7r5 cells with empty intracellular $Ca^{2+}$ stores

Our previous analyses of A7r5 cells established that AVP stimulates $Ca^{2+}$ entry via both a capacitative and a non-capacitative pathway, the latter almost certainly reflecting the activity of a non-selective cation channel [26]. Since the counteracting effects of AVP on $Ca^{2+}$ entry and extrusion would confuse

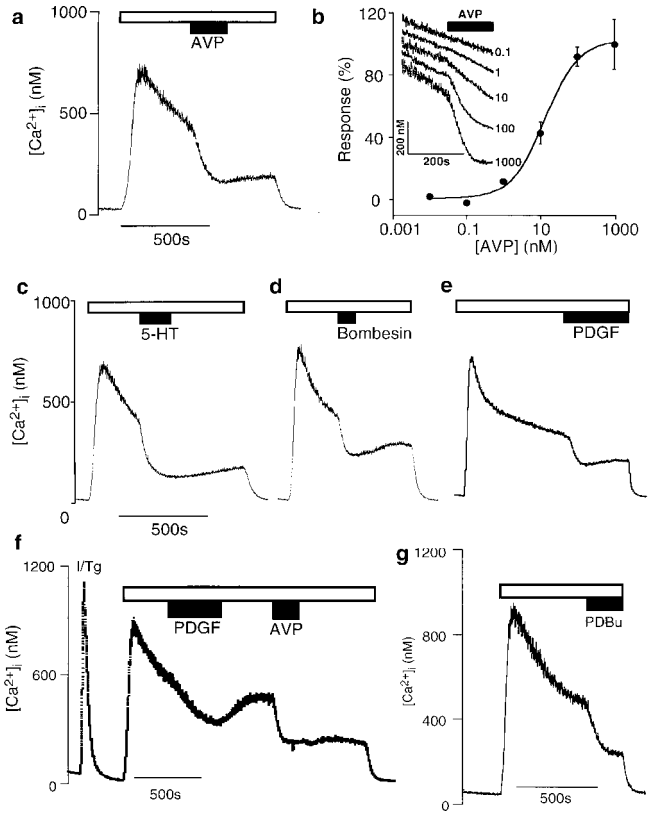


**Figure 1 Receptors linked to polyphosphoinositide hydrolysis evoke a decrease in $[Ca^{2+}]_i$ in cells where $[Ca^{2+}]_i$ has been increased by capacitative $Ca^{2+}$ entry**

A7r5 cells were pretreated with ionomycin and thapsigargin (I/Tg, 1 $\mu$M of each, 3 min; response shown only in **f**) in $Ca^{2+}$-free HBS to fully empty their intracellular stores; the same protocol was also used in all subsequent Figures. Extracellular $Ca^{2+}$ was restored for the periods shown by open bars and AVP (**a**, 100 nM), 5-HT (**c**, 50 $\mu$M), bombesin (**d**, 100 nM) or PDGF (**e**, 5 nM) were added for the periods shown by solid bars. The concentration-dependent effects of AVP on the decrease in $[Ca^{2+}]_i$ (mean $\pm$ S.E.M., $n = 3$) are shown in (**b**). The effect of PDGF (5 nM) on $Ca^{2+}$ recovery reverses rapidly, whereas the effect of AVP (100 nM) generally persists after its removal (**f**). (**g**) Activation of PKC by addition of PDBu (1 $\mu$M) also causes a decrease in $[Ca^{2+}]_i$. Traces are each representative of at least three similar experiments. In these and all subsequent experiments, A7r5 cells lacking hormone-regulated non-capacitative $Ca^{2+}$ entry were used.

analysis of the latter, we have taken advantage of a recently isolated stable variant of the A7r5 cell line in which the non-capacitative pathway is no longer regulated by hormones, although the other $Ca^{2+}$ transport pathways retain their normal sensitivities to hormonal regulation [4]. All subsequent experiments used the variant cell line in which hormones do not stimulate the non-capacitative pathway.

Addition of AVP (100 nM) to A7r5 cells in which $[Ca^{2+}]_i$ had been increased by capacitative $Ca^{2+}$ entry caused a concentration-dependent ($EC_{50} = 13 \pm 3$ nM, $n = 6$) decrease in $[Ca^{2+}]_i$ (Figure 1). This response is slightly more sensitive to AVP than is $Ca^{2+}$ mobilization ($EC_{50} = 32 \pm 5$ nM, $n = 3$). Despite the reduction in $[Ca^{2+}]_i$ evoked by AVP, $[Ca^{2+}]_i$ always remained elevated above the levels observed after removal of extracellular $Ca^{2+}$ (Figure 1). Other stimuli, including bombesin, 5-hydroxytryptamine (5-HT) and PDGF, each of which evokes release of intracellular $Ca^{2+}$ stores in A7r5 cells [4], also caused a reduction in the $[Ca^{2+}]_i$ of A7r5 cells during capacitative $Ca^{2+}$ entry (Figure 1). Similar effects have been observed in other cells [19,20]. It is important

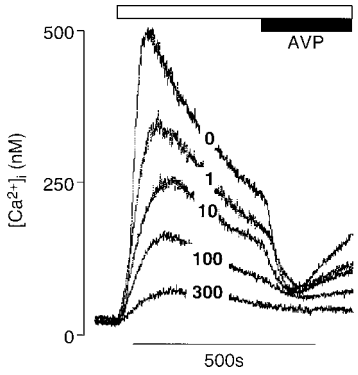


**Figure 2 AVP causes $[Ca^{2+}]_i$ to fall to similar level irrespective of the initial $[Ca^{2+}]_i$**

$[Ca^{2+}]_i$ was increased to various levels by restoring extracellular $Ca^{2+}$ (open bar) to cells with empty stores in the presence of the indicated concentrations (nM) of $Gd^{3+}$. Addition of AVP (1 $\mu$M, solid bar) caused $[Ca^{2+}]_i$ to fall to a similar level irrespective of the initial $[Ca^{2+}]_i$. Representative traces, typical of two experiments, are shown.

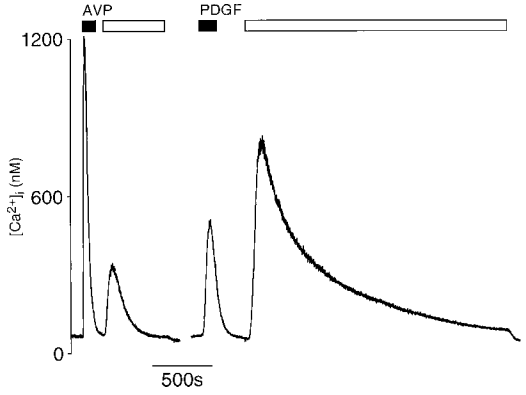


**Figure 3 Sustained and transient emptying of stores by PDGF and AVP respectively**

Cells were stimulated with AVP (100 nM) or PDGF (5 nM) in $Ca^{2+}$-free HBS for the periods shown by solid bars. The agonist was removed, extracellular $Ca^{2+}$ restored (open bars) and the subsequent changes in $[Ca^{2+}]_i$ recorded. The traces are each typical of at least three similar experiments.

to emphasise that while the variant cells are more amenable than normal A7r5 cells to analysis of these effects, the same stimuli also evoked decreases in $[Ca^{2+}]_i$ in the parental cell line [26] (results not shown). The decreases in $[Ca^{2+}]_i$ evoked by AVP, 5-HT and bombesin were rapid, well-maintained, and often ($\sim 70\%$ of experiments) reversed only slowly after removal of the agonist (Figures 1a, 1c and 1d). In contrast, the decrease in $[Ca^{2+}]_i$ evoked by PDGF was slower and generally reversed within 200 s of removing PDGF (Figures 1e and 1f). The effects of receptor stimulation were mimicked by addition of PDBu (1 $\mu$M) to activate PKC (Figure 1g), but not by its inactive analogue, 4$\alpha$-phorbol 12,13-dibutyrate (1 $\mu$M, results not shown).

$Gd^{3+}$, by irreversibly inhibiting capacitative $Ca^{2+}$ entry in A7r5 cells [4], allowed the increase in $[Ca^{2+}]_i$ evoked by the pathway to be manipulated without changing the extracellular $[Ca^{2+}]$. When $Gd^{3+}$ (1–1000 nM) was used to progressively reduce the extent to which $[Ca^{2+}]_i$ was elevated, AVP continued to cause a further reduction in $[Ca^{2+}]_i$ until, at a threshold $[Ca^{2+}]_i$ of about 100 nM, it failed to have any effect (Figure 2). Similar results were obtained when the initial $[Ca^{2+}]_i$ was adjusted using the cation channel blocker, LOE-908 (results not shown). This threshold $[Ca^{2+}]_i$ of $\sim 100$ nM was similar to the level to which AVP invariably caused $[Ca^{2+}]_i$ to fall (see Figure 6b).

To establish how rapidly the ability of PDGF and AVP to evoke $Ca^{2+}$ mobilization reversed after their removal from the bathing medium, cells were stimulated in $Ca^{2+}$-free HBS to empty the intracellular $Ca^{2+}$ stores and the agonist was then removed before restoration of extracellular $Ca^{2+}$. The duration of the ensuing $Ca^{2+}$ entry signal would be expected to persist only for as long as the Ins$P_3$ formed in response to receptor activation maintained the stores in a depleted state. The results demonstrate that the stores have refilled within 500 s of removing AVP (or bombesin or 5-HT), while even 2300 s after removal of PDGF they remain depleted (Figure 3).

These results indicate that whereas both PDGF and AVP cause a decrease in $[Ca^{2+}]_i$, after washout of the agonist the effect of AVP on $Ca^{2+}$ recovery generally persists long after it has ceased to stimulate $Ca^{2+}$ mobilization, while the effect of PDGF on $Ca^{2+}$ recovery reverses rapidly. It is difficult to accommodate these different patterns of reversal within a scheme involving either a single mechanism linking the two receptors to $Ca^{2+}$ recovery or a straightforward link between $Ca^{2+}$ recovery and stimulation of PLC.

## Stimulated $Ca^{2+}$ removal from the cytosol

$[Ca^{2+}]_i$ reflects the balance between $Ca^{2+}$ entering the cytosol (solely via capacitative $Ca^{2+}$ entry in our experiments) and the activities of the various processes that actively remove $Ca^{2+}$ from the cytosol. We therefore attempted to establish whether inhibition of $Ca^{2+}$ entry or stimulation of $Ca^{2+}$ removal accounted for the ability of physiological stimuli to cause a decrease in $[Ca^{2+}]_i$.

In A7r5 cells, both $Ba^{2+}$ and $Mn^{2+}$ permeate the capacitative pathway, but since neither is actively removed from the cytosol [26] they each provide reliable means of measuring the unidirectional entry of bivalent cations through the capacitative pathway. The enhanced rates of $Mn^{2+}$ and $Ba^{2+}$ entry, recorded after activation of the capacitative pathway by depletion of the intracellular $Ca^{2+}$ stores [26], were unaffected by either AVP (100 nM) or PDGF (5 nM) (Figure 4). Both agonists must therefore cause a decrease in $[Ca^{2+}]_i$ despite the unchanging activity of the capacitative pathway.

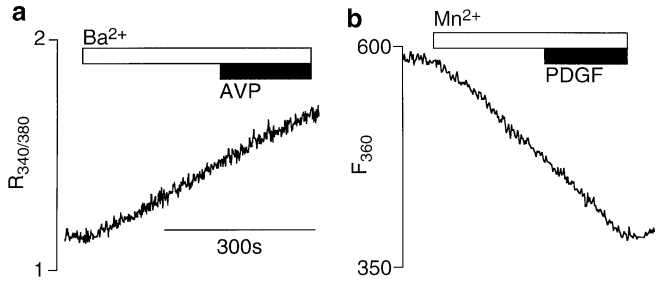


**Figure 4 AVP and PDGF do not inhibit capacitative $Mn^{2+}$ or $Ba^{2+}$ entry**

Cells with empty $Ca^{2+}$ stores were exposed to HBS containing $BaCl_2$ (**a**, 2 mM) or $MnCl_2$ (**b**, 100 $\mu$M) and the rates of bivalent cation entry were recorded by monitoring appropriate fura-2 excitation wavelengths; either the fluorescence ratio $R_{340/380}$ (**a**) or the fluorescence at a single excitation wavelength $F_{360}$ (**b**). AVP (100 nM) or PDGF (5 nM) were included for the periods shown (solid bars). Similar results were obtained when the combination of agonist and permeant cation was reversed. Results are typical of four independent experiments.

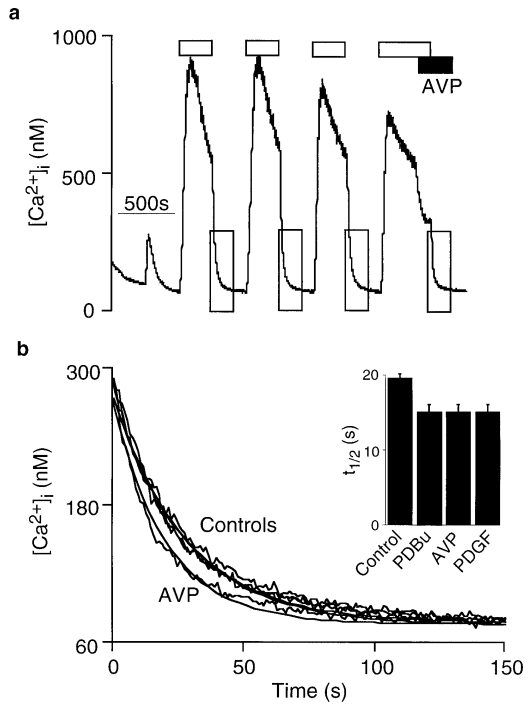


**Figure 5 AVP stimulates $Ca^{2+}$ extrusion**

Cells with empty $Ca^{2+}$ stores were repeatedly exposed to HBS containing extracellular $Ca^{2+}$ and, after $[Ca^{2+}]_i$ had increased, extracellular $Ca^{2+}$ was rapidly removed and the monoexponential rate of decline of $[Ca^{2+}]_i$ was recorded over the same range of $[Ca^{2+}]_i$ (vertical open boxes in **a**) for each recording. After three control recordings, the rate of fall of $[Ca^{2+}]_i$ was recorded after addition of AVP (100 nM) (final part of **a**). The declines in $[Ca^{2+}]_i$ before (Controls) and after stimulation with AVP, from which $t_{1/2}$ values were derived by fitting monoexponential equations, are shown in (**b**). The histogram shows results (unpaired comparisons with controls; the results shown in the text are paired comparisons) from similar experiments (means $\pm$ S.E.M., $n = 5$–19) with PDBu (1 $\mu$M), AVP (100 nM) and PDGF (5 nM).

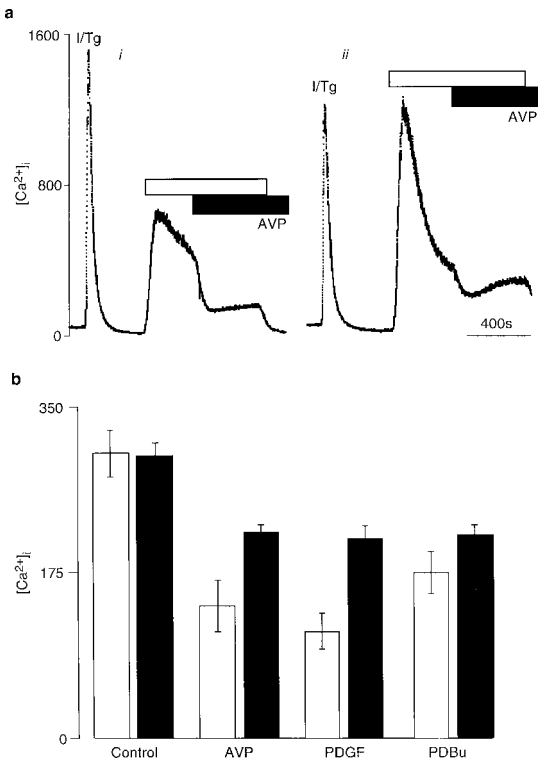


**Figure 6 Stimulation of $Ca^{2+}$ recovery in the absence of extracellular $Na^+$**

(**a**) Capacitative $Ca^{2+}$ entry was initiated by restoration of extracellular $Ca^{2+}$ (open bars) to cells with empty stores in either normal HBS (*i*) or $Na^+$-free HBS (*ii*), and AVP (100 nM, solid bars) was then added. The traces are each typical of at least seven independent experiments. (**b**) Results from experiments similar to those shown in the traces are summarized: cells were stimulated with AVP (100 nM) PDGF (2.5 nM) or PDBu (1 $\mu$M) in the presence (open bars) or absence (solid bars) of extracellular $Na^+$. Results [means $\pm$ S.E.M. of five (with $Na^+$) and seven (without $Na^+$) independent experiments] show the lowest $[Ca^{2+}]_i$ attained within 200 s of addition of the stimulus. The control columns ($n = 15$ for $Na^+$-containing; $n = 20$ for $Na^+$-free) show the estimated control $[Ca^{2+}]_i$ at the time at which the effects of the stimulus were determined extrapolated from the measurements preceding addition of the stimulus.

To directly quantify $Ca^{2+}$ removal from the cytosol, the monoexponential rate of decline of $[Ca^{2+}]_i$ after rapid removal of extracellular $Ca^{2+}$ was measured over the same range of $[Ca^{2+}]_i$ in the presence and absence of agonist. Under control conditions, removal of extracellular $Ca^{2+}$ from cells with fully depleted intracellular $Ca^{2+}$ stores caused $[Ca^{2+}]_i$ to decline to its basal level ($74 \pm 1$ nM) with a half-time ($t_{1/2}$) of $22 \pm 1$ s ($n = 35$). The rate of recovery was stable during repeated restoration and removal of extracellular $Ca^{2+}$: the $t_{1/2}$ was $21 \pm 1$ s, $21 \pm 1$ s and $22 \pm 1$ s ($n = 5$) after the first, second and third removal of extracellular $Ca^{2+}$ respectively (Figure 5a). In paired comparisons, where rates of $[Ca^{2+}]_i$ recovery were determined before and after stimulation (2 min) with AVP (100 nM), AVP caused the $t_{1/2}$ for recovery to decrease to $76 \pm 2\%$ ($n = 8$) of its control value (Figure 5b). In similar experiments with PDGF (5 nM) and PDBu (1 $\mu$M), the $t_{1/2}$ for recovery decreased to $84 \pm 4\%$ ($n = 7$) and $74 \pm 6\%$ ($n = 5$) of their control values respectively (Figure 5b).

## The extrusion mechanism

Ionomycin and thapsigargin ensure that $Ca^{2+}$ uptake into the intracellular stores cannot be the means whereby agonists decrease the $[Ca^{2+}]_i$, and the ability of AVP to reduce $[Ca^{2+}]_i$ to $< 100$ nM is inconsistent with the involvement of mitochondria, which accumulate $Ca^{2+}$ only when $[Ca^{2+}]_i$ is substantially higher [28]. A7r5 and vascular smooth muscle cells express an $Na^+$–$Ca^{2+}$ exchange system and it can be both phosphorylated and activated by PKC [29,30], but it cannot be the only means whereby receptors decrease $[Ca^{2+}]_i$. The peak amplitude of the $[Ca^{2+}]_i$ evoked by capacitative $Ca^{2+}$ entry was significantly higher when extracellular $Na^+$ ($723 \pm 69$ nM, $n = 4$) was replaced by *N*-methyl D-glucamine ($1111 \pm 59$ nM, $n = 8$) (Figure 6a) [29], although after 400 s (when agonists were added to assess their effects on $Ca^{2+}$ recovery), $[Ca^{2+}]_i$ had fallen to a similar level in the presence and absence of extracellular $Na^+$ (Figure 6b). Addition of maximally effective concentrations of AVP, PDGF or PDBu during this sustained elevation of $[Ca^{2+}]_i$ evoked significant decreases in $[Ca^{2+}]_i$ in the absence of extracellular $Na^+$, although the decreases were smaller than those recorded in the presence of extracellular $Na^+$ (Figure 6). Furthermore, while the $t_{1/2}$ for $[Ca^{2+}]_i$ recovery after rapid removal of extracellular $Ca^{2+}$ was increased from $25 \pm 2$ s to $31 \pm 4$ s ($n = 4$) by removal of extracellular $Na^+$, in paired comparisons AVP (100 nM) still caused the $t_{1/2}$ to fall to $85 \pm 5\%$ of its control value in the absence of $Na^+$. These results demonstrate that under conditions where $Na^+$–$Ca^{2+}$ counter-transport cannot have been active, each of the stimuli remains capable of stimulating $Ca^{2+}$ extrusion (Figure 6). Since removal of extracellular $Na^+$ is likely to cause hyperpolarization of the plasma membrane and to thereby increase the electrochemical gradient for $Ca^{2+}$ extrusion, the lesser effects of

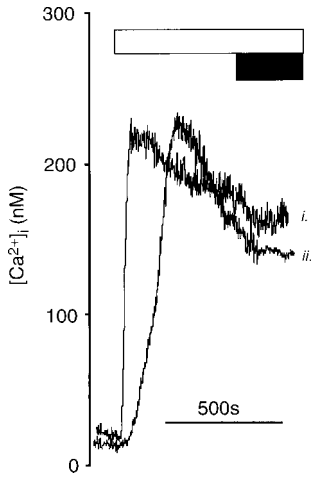


**Figure 7 Bis-phenol inhibits AVP-evoked $Ca^{2+}$ extrusion**

A7r5 cells were pre-treated with thapsigargin and ionomycin before addition of bis-phenol (30 $\mu$M), restoration of extracellular $Ca^{2+}$ (open bar) and then stimulation with AVP (1 $\mu$M, solid bar). The very slow and relatively modest rise in $[Ca^{2+}]_i$ in the presence of bis-phenol (*ii*) suggests that it may inhibit $Ca^{2+}$ entry as well as the plasma membrane $Ca^{2+}$ pump. The control trace (*i*) was selected to ensure that at the time of AVP addition, the $[Ca^{2+}]_i$ was similar (albeit lower than in most other traces) to that of the cells treated with bis-phenol. The results, typical of three experiments, show a modest decrease in $[Ca^{2+}]_i$ evoked by AVP in control cells (*i*) and no effect of AVP after treatment with bis-phenol (*ii*).

the stimuli in the absence of extracellular $Na^+$ is unlikely to be wholly attributable to the involvement of $Na^+$–$Ca^{2+}$ counter-transport. We conclude that the same agonists that cause $Ca^{2+}$ mobilization by stimulating formation of Ins$P_3$ also promote $Ca^{2+}$ recovery by stimulating the plasma membrane $Ca^{2+}$ pump. Experiments with extracellular $La^{3+}$ [17], eosin [31] or high pH, to inhibit the plasma membrane $Ca^{2+}$ pump directly, proved not to be useful, because each had additional side-effects. Bis-phenol has previously been reported to inhibit the $Ca^{2+}$ pumps of the endoplasmic reticulum [sarcoplasmic/endoplasmic-reticulum $Ca^{2+}$-ATPase (SERCA) and plasma membrane $Ca^{2+}$-ATPase (PMCA)] with similar potency [32]. Despite this lack of selectivity, bis-phenol could be used in our experiments because the SERCA were already fully inhibited by thapsigargin. The results (Figure 7) provide further evidence that the ability of AVP to stimulate a decrease in $[Ca^{2+}]_i$ is likely to be mediated by a plasma membrane $Ca^{2+}$ pump: 30 $\mu$M bis-phenol prevented the usual decrease in $[Ca^{2+}]_i$ evoked by AVP (1 $\mu$M).

## Role of PKC

In various cell types, activation of PKC increases the rate of $Ca^{2+}$ recovery [33] by stimulating either the plasma membrane $Ca^{2+}$ pump [16,24,34–36] or $Na^+$–$Ca^{2+}$ exchanger [29,30]. Our results with A7r5 cells are also consistent with PKC causing a decrease in $[Ca^{2+}]_i$ (Figure 1g) that is at least substantially due to activation of a plasma membrane $Ca^{2+}$ pump (Figure 6b). Subsequent experiments aimed to establish whether PKC provides the link between PDGF and AVP receptors and their effects on $Ca^{2+}$ extrusion.

Pretreatment of cells (15 min) with the selective inhibitor of PKC, Ro31-8220 (10 $\mu$M) [37], had no effect on capacitative $Ca^{2+}$ entry (Figure 8d), but completely inhibited the ability of PDBu ($\leqslant$ 10 $\mu$M) to cause a decrease in $[Ca^{2+}]_i$ (it fell by only $3 \pm 3$ nM, $n = 11$) (Figures 8a and 8d). However, subsequent addition of AVP (50 nM) caused a reduction in $[Ca^{2+}]_i$ (Figures 8a, 8b and 8d). Similar results were obtained when cells were stimulated with a much lower concentration of AVP (1 nM), which evoked a lesser fall in $[Ca^{2+}]_i$ ($54 \pm 18$ nM, $n = 8$) than that evoked by 5 nM PDGF or 1 $\mu$M PDBu (Figure 8d): Ro31-8220 still failed to inhibit the response evoked by AVP ($[Ca^{2+}]_i$ fell by $48 \pm 9$ nM, $n = 8$) while abolishing the responses to PDGF (the decrease was only $14 \pm 17$ nM, $n = 4$) and PDBu (Figure 8d). The $t_{1/2}$ for $Ca^{2+}$ removal from unstimulated cells was slightly increased (to $109 \pm 3\%$ of control, $n = 12$) after pretreatment with Ro31-8220 (10 $\mu$M, 15 min), consistent with some basal PKC activity [4]. However, Ro31-8220 abolished the effect of PDBu on the $t_{1/2}$ of $[Ca^{2+}]_i$ recovery ($105 \pm 9\%$ of control, $n = 4$) without preventing the decrease evoked by AVP ($87 \pm 5\%$ of control, $n = 9$). Ro31-8220 also failed to prevent the stimulation of $Ca^{2+}$ efflux evoked by 5-HT. Finally, staurosporine, at a concentration (1 $\mu$M) that fully inhibited the response to PDBu, had no effect on the response to AVP (results not shown).

These results establish that AVP stimulates $Ca^{2+}$ extrusion by a mechanism that does not depend on PKC. The effects of Ro31-8220 (10 $\mu$M) on the responses to PDGF are more difficult to interpret, because while Ro31-8220 had no effect on AVP-evoked $Ca^{2+}$ mobilization, it substantially inhibited PDGF-evoked $Ca^{2+}$ mobilization (Figure 8c, d). It proved impossible to select a concentration of Ro31-8220 that inhibited PKC without similarly attenuating PDGF-evoked $Ca^{2+}$ mobilization. Chronic exposure to phorbol esters causes down-regulation of PKC, but this approach was also unsuccessful because treatment of cells with PDBu (1–10 $\mu$M) for 3 h reduced the capacitative $Ca^{2+}$ entry signal such that $[Ca^{2+}]_i$ was too low to allow the effects of hormones on $Ca^{2+}$ efflux to be determined.

## Role of PLC

The results so far indicate that while PKC can stimulate $Ca^{2+}$ extrusion, it is not the means whereby AVP is linked to the $Ca^{2+}$ efflux pathway. In leukocytes too, PKC stimulates $Ca^{2+}$ recovery, but appears not to be the mechanism recruited by chemoattractant peptide [38]. We next attempted to establish whether stimulation of PLC was necessary for PDGF or AVP to stimulate $Ca^{2+}$ extrusion.

The only available membrane-permeant inhibitor of PLC, U73122 [39], also directly inhibits capacitative $Ca^{2+}$ entry [4,40] and thereby causes a decrease in $[Ca^{2+}]_i$ such that in many experiments $[Ca^{2+}]_i$ fell to too low a level to allow the effects of stimuli on $Ca^{2+}$ extrusion to be resolved (Figure 9b, *ii*). However, in experiments where $[Ca^{2+}]_i$ remained sufficiently high after incubation with U73122 (10–30 $\mu$M), addition of AVP (100 nM, Figure 9d) or PDGF (2.5 nM, Figure 9b*i*) still caused a decrease in $[Ca^{2+}]_i$. Both our previous work [4] and parallel experiments indicate that under these conditions U73122 completely abolished both Ins$P_3$ formation ($IC_{50} = 0.9 \pm 0.1$ $\mu$M) and PDGF- or AVP-evoked mobilization of intracellular $Ca^{2+}$ stores (Figure 9a).

In an attempt to separate more clearly the inhibitory effects of U73122 on PLC and the capacitative pathway, we exploited our observation that even 30 min after removal of U73122, there was no reversal of the inhibition of PDGF-evoked $Ca^{2+}$ mobilization (Figure 9a, *ii*). The results are shown in Figure 9(c) and again demonstrate that PDGF stimulates a decrease in $[Ca^{2+}]_i$ even when PLC had been inhibited by prior incubation with U73122. We conclude that the ability of both AVP and PDGF to stimulate $Ca^{2+}$ extrusion does not require stimulation of PLC.

Arachidonic acid stimulates the plasma membrane $Ca^{2+}$ pump of macrophages [23]. The same mechanism is unlikely to be involved in A7r5 cells, because stimuli that caused a decrease in $[Ca^{2+}]_i$ failed to stimulate the non-capacitative $Ca^{2+}$ entry path-

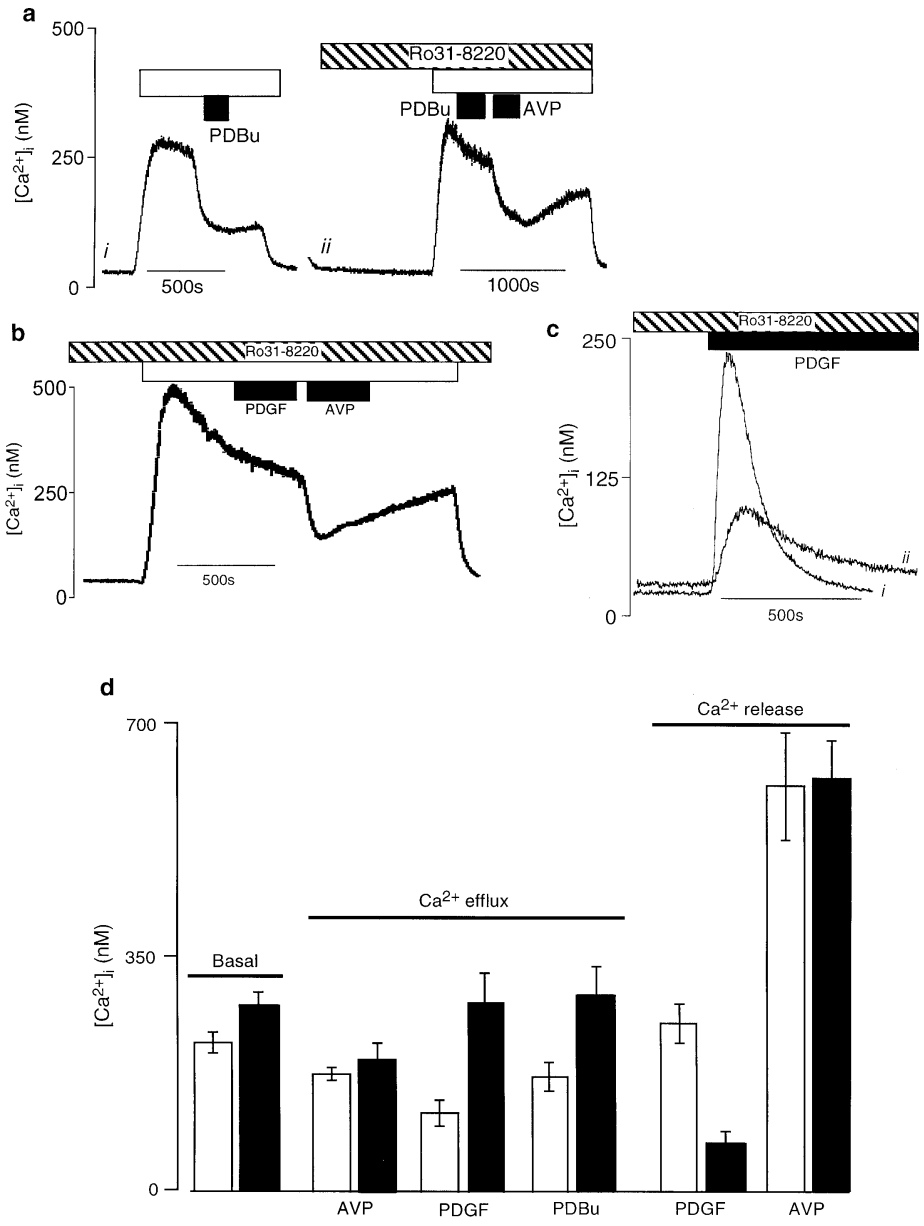


**Figure 8 Effects of inhibiting PKC on PDGF- and AVP-evoked $Ca^{2+}$ efflux**

(**a**) Addition of PDBu (1 $\mu$M, solid bar) to A7r5 cells in which $[Ca^{2+}]_i$ had been elevated by capacitative $Ca^{2+}$ entry (open bar) caused a rapid drop in $[Ca^{2+}]_i$ (*i*) that was prevented by Ro31-8220 (10 $\mu$M, hatched bar) (*ii*). (**b**) The response to AVP (10 nM) was unaffected by Ro31-8220 (**a***ii*, **b**), whereas that to PDGF (5 nM) was abolished (**b**). (**c**) The release of intracellular $Ca^{2+}$ stores following stimulation with PDGF (5 nM) in $Ca^{2+}$-free HBS was substantially reduced by Ro31-8220 (10 $\mu$M, hatched bar) (*ii*). Results show representative traces, each typical of at least three similar experiments. (**d**) The effects of Ro31-8220 (solid bars) on the drop in $[Ca^{2+}]_i$ ('$Ca^{2+}$ efflux') evoked by PDBu, PDGF or AVP are shown as means $\pm$ S.E.M. of at least four independent experiments. The first columns show the control $[Ca^{2+}]_i$ at the time at which the response to agonist was measured ($n = 15$ and 20) (see legend to Figure 6) and the last columns show the effects of Ro31-8220 on PDGF- and AVP-evoked release of intracellular $Ca^{2+}$ stores ($n = 3$).

way which was previously shown to be regulated by arahidonic acid [4]. We conclude, in keeping with the lack of effect of RHC 80267 (50 $\mu$M), an inhibitor of diacylglycerol lipase, on AVP-evoked $Ca^{2+}$ extrusion (results not shown), that arachidonic acid is unlikely to mediate stimulation of the plasma membrane $Ca^{2+}$ pump in A7r5 cells.

## Conclusions

In A7r5 cells, each of the agonists that stimulates Ins$P_3$-evoked $Ca^{2+}$ mobilization also promotes $Ca^{2+}$ recovery by stimulating $Ca^{2+}$ extrusion across the plasma membrane by a mechanism that does not require extracellular $Na^+$ and probably, therefore, results from stimulation of the plasma membrane $Ca^{2+}$ pump. The ability of the stimulated recovery mechanism to reduce $[Ca^{2+}]_i$ to $\sim$ 100 nM and its sensitivity to bis-phenol are also consistent with the plasma membrane $Ca^{2+}$ pump being the major target [34]. Phosphorylation of the plasma membrane $Ca^{2+}$ pump by PKC increases its activity [34] and our results are consistent with a similar mechanism operating in A7r5 cells. However, PKC is not the means whereby AVP (or other agonists of G-protein-coupled receptors) stimulate $Ca^{2+}$ extrusion, nor is

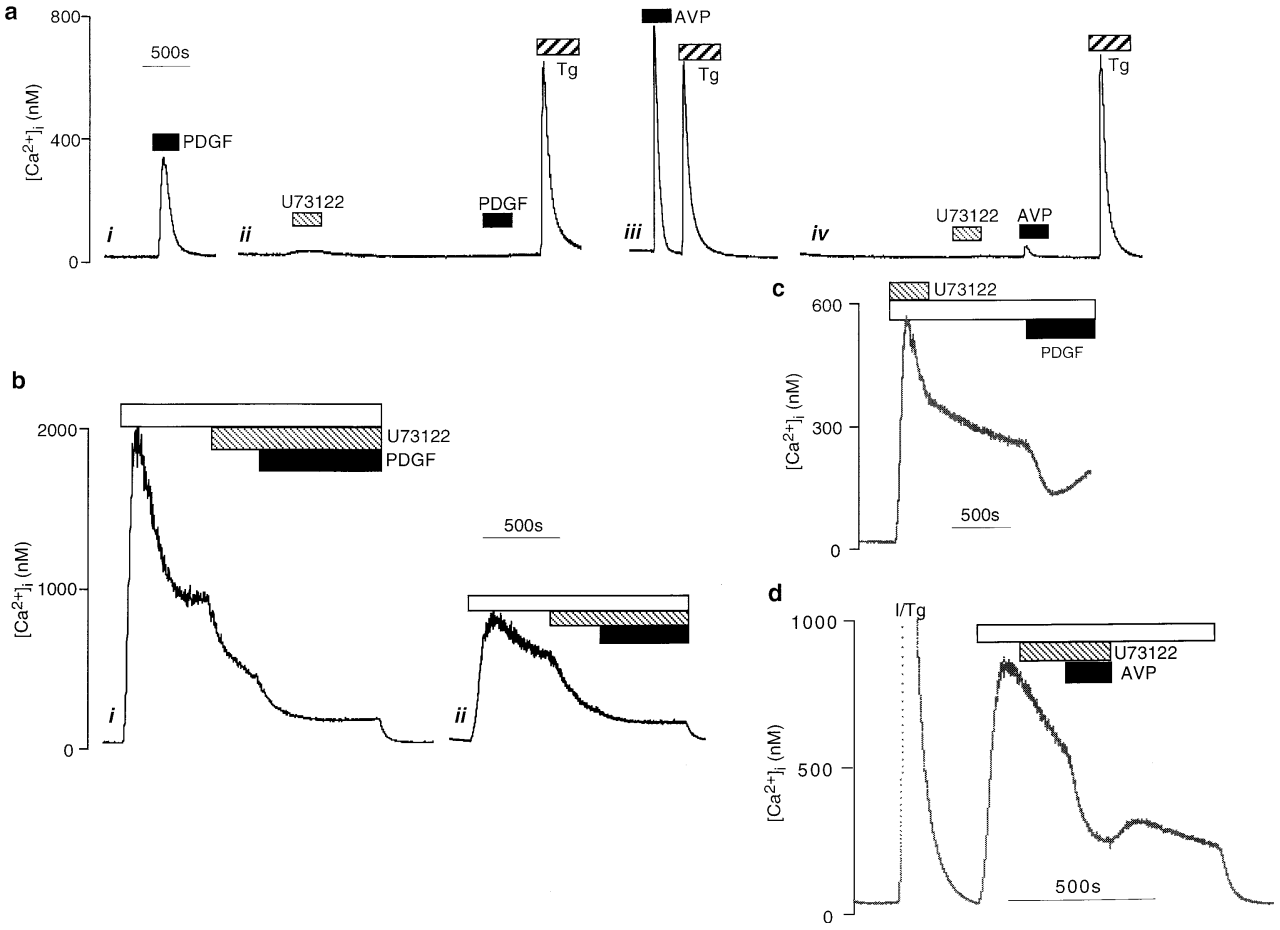


**Figure 9 Effects of inhibiting PLC on PDGF- and AVP-evoked $Ca^{2+}$ efflux**

(**a**) A7r5 cells were stimulated with PDGF (2.5 nM, *i* and *ii*) or AVP (20 nM, *iii* and *iv*) in $Ca^{2+}$-free HBS and the responses were compared with cells that had been pre-treated with U73122 (5 min, 10 $\mu$M *ii* and *iv*). Thapsigargin (Tg) was then added to establish the $Ca^{2+}$ content of the intracellular stores. The results show that even 30 min after washout of U73122 (*ii*), receptor-stimulated PLC remains fully inhibited. Results are typical of at least three similar experiments. Similar inhibition was observed when U73122 was added only 2 min before addition of agonist. (**b**) PDGF (2.5 nM, solid bars) caused a rapid decrease in $[Ca^{2+}]_i$ in the presence of U73122 (10 $\mu$M, hatched bars) (*i*) provided that the inhibition of capacitative $Ca^{2+}$ entry by U73122 did not cause $[Ca^{2+}]_i$ to fall below the level at which agonists fail to cause a further reduction (*ii*). (**c**) Pretreatment with U73122 (10 $\mu$M, hatched bar) allowed its direct effects on $Ca^{2+}$ entry to be separated from the response to PDGF (2.5 nM, solid bar). (**d**) The response to AVP (100 nM, solid bar) persists in the presence of U73122 (10 $\mu$M, hatched bar).

it likely to be the means whereby PDGF stimulates $Ca^{2+}$ extrusion. Instead, and in keeping with the very different time courses over which $Ca^{2+}$ extrusion and Ins$P_3$-mediated $Ca^{2+}$ mobilization reverse after removal of the agonist, both families of receptors stimulate $Ca^{2+}$ extrusion by a mechanism that appears not to require activation of PLC. Plasma membrane $Ca^{2+}$ pumps are extensively regulated, with calmodulin, proteases, acidic phospholipids, phosphorylation, G-proteins and oligomerization all implicated in controlling different isoforms [18]. At present, we can only speculate on the likely links between the PDGF and AVP receptors and stimulation of the plasma membrane $Ca^{2+}$ pump in A7r5 cells. We cannot yet completely eliminate a role for PKC in mediating the effects of the PDGF receptor, although phosphorylation of a tyrosine residue on the $Ca^{2+}$ pump is another possibility [18]. A possible explanation for the effects of AVP, and presumably the other agonists of G-protein-linked receptors, is that the plasma membrane $Ca^{2+}$ pump may be directly stimulated by G-protein subunits.

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