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Distinct Ca^{2+} signalling mechanisms induced by ATP and sphingosylphosphorylcholine in porcine aortic smooth muscle cells

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1 The increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) following repetitive stimulation with ATP or sphingosylphosphorylcholine (SPC) in single porcine aortic smooth muscle cells was investigated using the Ca^{2+} indicator, fura-2.

2 The ATP-induced $[Ca^{2+}]_i$ increase resulted from both Ca^{2+} release and Ca^{2+} influx. The former was stimulated by phospholipase C activation, while the latter occurred predominantly *via* the receptor-operated Ca^{2+} channels (ROC), rather than the store-operated Ca^{2+} channels (SOC) or the voltage-operated Ca^{2+} channel (VOC). Furthermore, the $P2X_5$ receptor was shown to be responsible for the ATP-induced Ca^{2+} influx.

3 A reproducible $[Ca^{2+}]_i$ increase was induced by repetitive ATP stimulation, but was abolished by removal of extracellular Ca^{2+} or inhibition of intracellular Ca^{2+} release using U-73122 or thapsigargin, and was restored by Ca^{2+} readdition in the former case.

4 SPC only caused Ca^{2+} release, and the amplitude of the repetitive SPC-induced $[Ca^{2+}]_i$ increases declined gradually. However, a reproducible $[Ca^{2+}]_i$ increase was seen in cells in which protein kinase C being inhibited, which increased the SPC-induced Ca^{2+} influx, rather than IP_3 generation.

5 In conclusion, although the amplitude of the ATP-induced Ca^{2+} release, measured when Ca^{2+} influx was blocked, or of the Ca^{2+} influx when Ca^{2+} release was blocked, progressively decreased following repetitive stimulation, the overall $[Ca^{2+}]_i$ increase for each stimulation under physiological conditions remained the same, suggesting that the Ca^{2+} stores were replenished by an influx of Ca^{2+} during stimulation. The SPC-induced $[Ca^{2+}]_i$ increase resulted solely from Ca^{2+} release and decreased gradually following repetitive stimulation, but the decrease could be prevented by stimulating Ca^{2+} influx, further supporting involvement of the intracellular Ca^{2+} stores in Ca^{2+} signalling.

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Keywords: Purinoceptor; sphingosylphosphorylcholine; cytosolic Ca^{2+} concentration; aortic smooth muscle cells; desensitization; receptor-operated Ca^{2+} channel; store-operated Ca^{2+} channel; voltage-operated Ca^{2+} channel

Abbreviations: $AACOCF_3$, arachidonyl trifluoromethyl ketone; AMPCPP, α,β-methylene adenosine 5′-triphosphate; ATPγS, adenosine 5′-O-(3-thiotriphosphate); BzATP, 2′- and 3′-*O*-(4-benzoylbenzoyl)-adenosine 5′-triphosphate; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; DIDS, 4,4′-diisothiocyano-2,2′-disulfonic acid stilbene; IP_3, inositol 1,4,5-trisphosphate; KN-62, {1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine}; 2-MeSATP, 2-methylthioadenosine 5′-triphosphate; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid; ROC, receptor-operated Ca^{2+} channel; Rp-cAMPS, Rp-adenosine 3′,5′-cyclic monophosphothioate; SK&F96365, 1-[β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl]-1H-imidazole HCl; SPC, sphingosylphosphorylcholine; SOC, store-operated Ca^{2+} channel; U-73122, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]-amino]hexyl]-1H-pyrrole-2,5-dione; VOC; voltage-operated Ca^{2+} channel

Introduction

Intracellular Ca^{2+} levels ($[Ca^{2+}]_i$) regulate many cellular functions, including secretion, contraction, neurotransmission, proliferation and apoptosis, and cells have therefore evolved distinct mechanisms to rapidly and precisely control the Ca^{2+} signalling process (Berridge, 1997). In the response to a receptor agonist, Ca^{2+} release from intracellular Ca^{2+} stores (*via* IP_3 generation) and Ca^{2+} influx *via* receptor-operated Ca^{2+} channels (ROC) are two distinct mechanisms responsible for the increased $[Ca^{2+}]_i$ (Marks, 1997; Furuichi & Mikoshiba, 1995; Barnard, 1996), while Ca^{2+} influx *via* voltage-operated Ca^{2+} channels (VOC) is predominantly responsible for the increase in the $[Ca^{2+}]_i$ seen on membrane depolarization (Hess, 1990). In addition to the IP_3-mediated Ca^{2+} release, intracellular Ca^{2+} release can be triggered by many other messengers, including GTP (Chueh & Gill, 1986), cyclic ADP ribose (Lee *et al*., 1989), arachidonic acid (Wu *et al*., 1994), sphingolipid metabolites (Ghosh *et al*., 1990), and nicotinic acid adenine dinucleotide phosphate (Dousa *et al*., 1996). It has also been shown that the permeability of the plasma membrane to Ca^{2+} increases after depletion of the intracellular Ca^{2+} stores by second messengers (Birnbaumer *et al*., 1996). The Ca^{2+} influx pathway linked to a decreased Ca^{2+} content of the stores has been termed capacitative Ca^{2+} entry *via* so-called store-operated Ca^{2+} channels (SOC) (Putney, 1990); however, the mechanism by which this occurs is unknown.

In many tissues, extracellular ATP levels act as a signal for the induction of various cellular responses *via* activation of a distinct receptor (Harden *et al*., 1995). In neuronal and muscle cells, ATP activates a nonselective cation channel (P_{2x} receptor) that is permeable to Ca^{2+} and Na^+ (Surprenant *et al*., 1995; Lewis *et al*., 1995). In many tissues, activation of P_{2y} or P_{2u} receptors is directly coupled to G proteins, with subsequent modulation of the activities of two effector

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systems, phospholipase and adenylyl cyclase. Phospholipases A_2, C and D have all been reported to be stimulated by extracellular ATP in different cell types (Wilkinson *et al*., 1993; Winitz *et al*., 1994), while the generation of cytosolic cyclic AMP is either inhibited or increased by the same stimulation (Sipma *et al*., 1994; Sato *et al*., 1992). In platelets, ADP, but not ATP, is the active agonist of P_{2t} receptors, activation of which stimulates Ca^{2+} release from intracellular Ca^{2+} stores *via* phospholipase C activation (Hourani & Hall, 1994). In the inflammatory and immune systems, activation of P_{2z} receptors opens nonselective pores which allows molecules with molecular masses of less than 1000 daltons to pass freely (Surprenant *et al*., 1996). Of the above mentioned ATP signalling cascades, activation of nonselective cation channels (P_{2x} receptor), nonselective pores (P_{2z} receptor), or phospholipase C (*via* activation of P_{2y}, P_{2u} or P_{2t} receptors) results in an increase in the $[Ca^{2+}]_i$.

Sphingolipid metabolites, including sphingosine 1-phosphate (S1P) and sphingosylphosphorylcholine (SPC), have been shown to act as extracellular signals (Meyer zu Heringdorf *et al*., 1997). Although the gene product of *edg*-1 has been identified as a S1P receptor (Lee *et al*., 1998), no SPC receptor has yet been cloned. Nevertheless, SPC transduces its signal *via* receptor-G protein-effector system coupling, since all SPC-stimulated signalling events are inhibited by pertussis toxin (Okajima & Kondo, 1995; Seufferlein & Rozengurt, 1995; van Koppen *et al*., 1996). Recently, we have shown that, in porcine aortic smooth muscle cells, the $[Ca^{2+}]_i$ can be increased by G protein-coupled receptor agonists, including ATP, bradykinin, SPC, and lysophosphatidic acid (LPA), but only SPC and LPA are mitogenic and their effects sensitive to pertussis toxin. We further demonstrated that SPC-induced mitogen-activated protein kinase activation depends on SPC-induced intracellular Ca^{2+} release *via* IP_3 generation and is not affected by the removal of extracellular Ca^{2+}. Platelet-derived growth factor also increases the $[Ca^{2+}]_i$, but its mitogenic effect is insensitive to $[Ca^{2+}]_i$ change (Chin & Chueh, 1998).

In the current study, again using porcine aortic smooth muscle cells, we further characterized the desensitization mechanisms involved in the $[Ca^{2+}]_i$ increase induced by repetitive ATP or SPC stimulation. The Ca^{2+} signals remained at the same level throughout repetitive ATP stimulation, while those induced by SPC gradually declined with repetitive stimulation. Our data indicate that replenishment of the intracellular Ca^{2+} stores by influxed Ca^{2+} plays a pivotal role in maintaining the reproducible Ca^{2+} signalling.

Methods

Materials

Dulbecco's modified Eagle's medium, foetal bovine serum and trypsin/EDTA were purchased from Life Technologies (Grand Island, NY, U.S.A.). Fura-2 acetoxymethyl ester was obtained from Molecular Probes (Eugene, OR, U.S.A.). ATP, DIDS, BzATP, ATPγS, SPC, verapamil, ω-conotoxin, and nifedipine were purchased from Sigma (St. Louis, MO, U.S.A.). Thapsigargin, Rp-cAMPS, PPADS, AMPCPP, 2-MeSATP and U-73122 were obtained from Research Biochemicals International (Natick, MA, U.S.A.). Arachidonyl trifluoromethyl ketone ($AACOCF_3$), KN-62, Gö 6983 and KT5823 were purchased from Calbiochem (San Diego, CA, U.S.A.). SK&F96365 was purchased from Biomol (Plymouth Meeting, PA, U.S.A.). The [^{3}H]-IP_3 assay system was obtained from the Amersham Corp (Buckinghamshire, U.K.). All other chemicals were analytical grade and obtained from Merck (Darmstadt, Germany).

Culture of porcine aortic smooth muscle cells

Porcine aortic smooth muscle cells were prepared using the explant method of Ross (1971), as previously described (Song & Chueh, 1996; Chin & Chueh, 1998). Explants of porcine aorta, obtained from a local slaughterhouse, were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% foetal bovine serum, 100 units ml^{-1} of penicillin, and 100 μg ml^{-1} of streptomycin, and maintained at 37°C in an atmosphere of 95% air and 5% CO_2. Over the next 10 days, the smooth muscle cells slowly migrated over the dish surface. After reaching confluency, they were subcultured by incubation with Ca^{2+}- and Mg^{2+}-free 0.05% trypsin/EDTA solution and maintained for five passages in culture. For $[Ca^{2+}]_i$ measurement and Mn^{2+} influx experiments, cells were plated onto glass coverslips 48 h before use. In some experiments, cells were preincubated with pertussis toxin (100 ng ml^{-1}) or cholera toxin (1 μg ml^{-1}) for 16 h.

Measurement of $[Ca^{2+}]_i$

The $[Ca^{2+}]_i$ change in a single cell was measured using the fluorescent Ca^{2+} indicator, fura-2, as described previously (Chin & Chueh, 1998). The $[Ca^{2+}]_i$ was calculated from the ratio of the fluorescence at 340 nm and 380 nm according to the equation derived by Grynkiewicz *et al*. (1985) using parameters obtained on our instrument for fura-2 in aortic smooth muscle cells: $R_{min}=1.18$; $R_{max}=3.5$; $S_{f2}/S_{b2}=1.22$; $K_d=135$ nM. In some experiments performed in the absence of extracellular Ca^{2+}, extracellular Ca^{2+} was omitted from the loading buffer and 0.5 mM EGTA was added after fura-2 loading. The loading buffer consisted of (mM): NaCl 150, KCl 5, glucose 5, $MgCl_2$ 1, $CaCl_2$ 2.2 and HEPES 10, pH 7.4. The results of one representative experiment are illustrated in the figures, and the mean$\pm$s.d. values for the $[Ca^{2+}]_i$ changes, calculated for *n* experiments using different batches of cells, are given in the text.

Measurement of IP_3 production

After near-confluency was reached in the six-well plates, the cells were pretreated with either buffer or 0.1 μM staurosporine for 5 min, then washed and incubated with the indicated agonists at 37°C for the indicated times (15 s to 4 min) in a volume of 1 ml of loading buffer per well. The amount of IP_3 generated in each well was determined by radioreceptor assay using the D-*myo*[^{3}H]-IP_3 assay system (Amersham) according to the manufacturer's instructions. The data presented are the means$\pm$s.d. for six independent experiments using different batches of cells.

Determination of fluorescence quenching by Mn^{2+} influx

Fura-2-loaded cells were bathed in loading buffer containing 1 mM $CaCl_2$. Where indicated, the cells were pretreated with (μM): SK&F96365 30, staurosporine 0.1, Rp-cAMPS 30, Gö 6983 0.3, KN-62 10 or KT5823 1 at room temperature for 5 min. At the indicated times, 1 mM Mn^{2+}, either alone or together with thapsigargin, ATP, or SPC, was added. The Mn^{2+} influx into the cells was monitored by quenching of fura-2 fluorescence at the isosbestic wavelength of 360 nm. All

experiments were repeated at least nine times using different batches of cells with similar results. The results for one representative experiment are shown in the figures.

Results

In this study, we first characterized the Ca^{2+} signalling mechanism induced by the purinoceptor agonist, ATP, in single porcine aortic smooth muscle cells. As shown in Figure 1A (trace a), the $[Ca^{2+}]_i$ increased rapidly from the basal level of 50 ± 17 nM ($n=76$) to a peak level of 729 ± 85 nM ($n=76$) within 5 s of the addition of 30 μM ATP in the presence of extracellular Ca^{2+}, then fell to the basal level over the next 180 s. When the extracellular Ca^{2+} was removed and 0.5 mM EGTA added to the bathing solution, this increase was reduced by approximately 38% (Figure 1A, trace b), the basal and peak levels of $[Ca^{2+}]_i$ being 42 ± 19 nM and 475 ± 30 nM ($n=68$), respectively, and the $[Ca^{2+}]_i$ fell to the basal level within 90 s. Figure 1B shows the dependence of the $[Ca^{2+}]_i$ changes, either at the peak (panel a) or 60 s after ATP addition (panel b), on the concentration of ATP. The difference between the ATP-induced $[Ca^{2+}]_i$ increase in the presence and absence of extracellular Ca^{2+} (open and filled circles, respectively) reflects the ATP-induced Ca^{2+} influx. These ATP-induced $[Ca^{2+}]_i$ increases were mediated by a purinoceptor, since they were inhibited by approximately 46–68% by pretreatment of the cells with 30 μM PPADS or 30 μM DIDS (data not shown).

In the presence of extracellular Ca^{2+}, the amplitude of the $[Ca^{2+}]_i$ increases induced by repetitive ATP challenge was not significantly reduced (Figure 2A, trace a). However, in the absence of extracellular Ca^{2+}, desensitization occurred, as the amplitude of $[Ca^{2+}]_i$ increase progressively decreased (Figure 2A, trace b). The data for the reduction in the $[Ca^{2+}]_i$ increase induced by repetitive ATP stimulation in the presence or absence of extracellular Ca^{2+} are summarized in Figure 2B, in

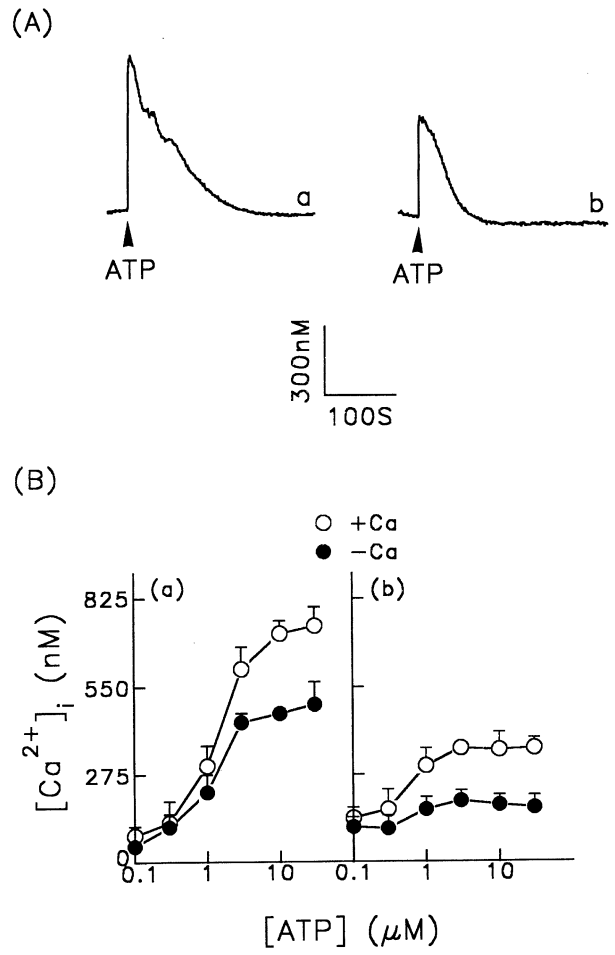

Figure 1 ATP-induced changes in $[Ca^{2+}]_i$ in porcine aortic smooth muscle cells. (A) ATP (30 μM) was added as indicated by the arrowheads to fura-2-loaded cell in the presence (trace a) or absence (trace b) of extracellular Ca^{2+}. (B) Various concentrations of ATP were added to cells in the presence or absence of extracellular Ca^{2+}. The $[Ca^{2+}]_i$ peak height (panel a) or the $[Ca^{2+}]_i$ increase 60 s after ATP addition (panel b) are plotted versus the ATP concentration. The data are the mean ± s.d. for 68–76 cells.

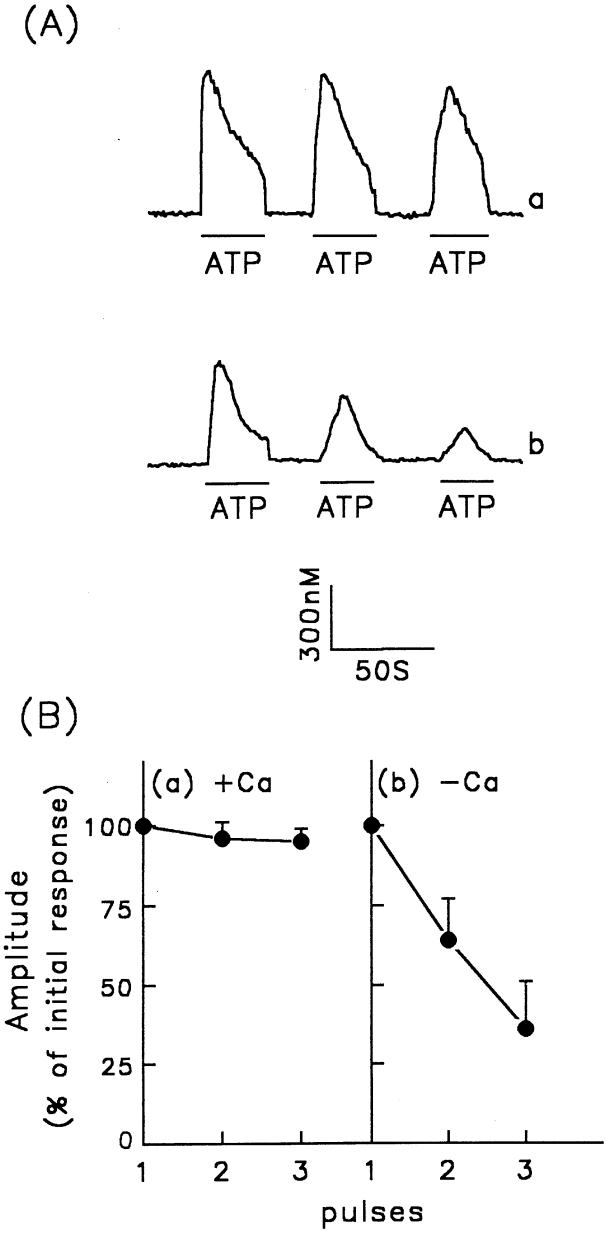

Figure 2 $[Ca^{2+}]_i$ increases induced by repetitive ATP stimulation in porcine aortic smooth muscle cells. (A) $[Ca^{2+}]_i$ changes in fura-2-loaded cells were measured in response to repetitive stimulation with 30 μM ATP as indicated by the horizontal bars in the presence (trace a) or absence (trace b) of extracellular Ca^{2+}. (B) Summarized data for the $[Ca^{2+}]_i$ changes induced by repetitive ATP stimulation in the presence (panel a) or absence (panel b) of extracellular Ca^{2+} under the conditions described above. The amplitudes of the $[Ca^{2+}]_i$ changes are expressed as the peak height for response n relative to that for the initial response. The values are the mean ± s.d. for 86–96 cells.

which the responses are expressed as the ratio of the response to the *n*th stimulus over that to the initial stimulus.

Our data indicate that both intracellular Ca^{2+} release and extracellular Ca^{2+} influx contribute to the Ca^{2+} signal induced by ATP in porcine aortic smooth muscle cells. The reduction in the $[Ca^{2+}]_i$ increase seen on repetitive stimulation in the absence of extracellular Ca^{2+} further indicates either that releasable Ca^{2+} was not available due to depletion of the intracellular Ca^{2+} stores after repetitive stimulation or that the phospholipase C signalling cascade was desensitized. We therefore examined the amount of available Ca^{2+} remaining within the intracellular Ca^{2+} stores after repetitive exposure to ATP, using the amplitude of the $[Ca^{2+}]_i$ transient induced by ionomycin in the absence of extracellular Ca^{2+} as an index of the size of the intracellular Ca^{2+} stores (Montero *et al*., 1990). As shown in Figure 3A, after repetitive ATP stimulation, the $[Ca^{2+}]_i$ transient induced by ionomycin was significantly less in the cells that had been subjected to ATP stimulation in Ca^{2+}-free buffer (trace a) than in those stimulated in Ca^{2+}-containing buffer (trace b), the peak levels of the $[Ca^{2+}]_i$ increases being 170 ± 18 ($n=46$) and 704 ± 25 ($n=46$), respectively. The available Ca^{2+} remaining within the intracellular Ca^{2+} stores was not reduced following repetitive ATP stimulation in the presence of extracellular Ca^{2+}, since the EGTA and ionomycin-induced $[Ca^{2+}]_i$ increase was identical to that for control cells not previously subjected to ATP challenge (trace c). Our results therefore suggest that the decreased response to ATP seen in Ca^{2+}-free medium is at least partially due to the filling state of the intracellular Ca^{2+} stores. Indeed, when Ca^{2+} was subsequently added back to the bathing solution after repetitive ATP stimulation in the absence of extracellular Ca^{2+}, reproducible ATP-induced $[Ca^{2+}]_i$ increases were fully restored, regardless of pretreatment with staurosporine (Figure 3B, traces a and b).

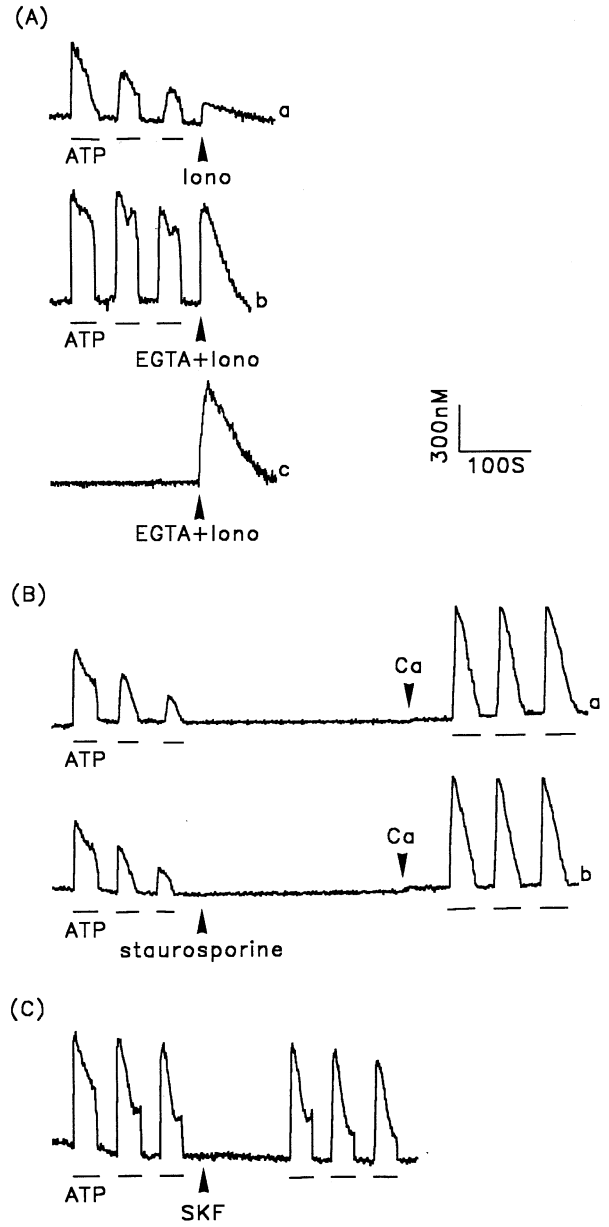

Figure 3 The effect of the Ca^{2+} content of the intracellular Ca^{2+} stores on repetitive ATP-induced $[Ca^{2+}]_i$ changes in porcine aortic smooth muscle cells. (A) The extent of filling of the intracellular Ca^{2+} stores in cells after repeated exposure to ATP in the absence (trace a) or presence (trace b) of extracellular Ca^{2+} and in control cells without ATP stimulation (trace c) was measured by the addition of 10 μM ionomycin only (Iono) (trace a), or 5 mM EGTA and 10 μM ionomycin (EGTA + Iono) (traces b and c), as shown by the arrowheads. (B) $[Ca^{2+}]_i$ changes induced by repetitive ATP stimulations were initially measured in the absence of extracellular Ca^{2+}. The cells shown in (a) were left untreated while those in (b) were treated with 0.1 μM staurosporine, then 2.2 mM $CaCl_2$ were reintroduced into the bathing solution in both cases, as indicated by the arrowheads, and the $[Ca^{2+}]_i$ changes induced by repetitive ATP stimulation again measured. (C) $[Ca^{2+}]_i$ changes induced by repetitive ATP stimulation in the presence of extracellular Ca^{2+} were measured before, and after, the introduction of 30 μM SK&F96365 (SKF), as shown by the arrowhead. ATP (30 μM) was applied as indicated by the horizontal bars. The experiments were performed 46–58 times with similar results; one representative example is shown.

Our data indicate that the ATP-induced $[Ca^{2+}]_i$ increase is attributable to both intracellular Ca^{2+} release and extracellular Ca^{2+} influx. Since ROC, VOC, and SOC are three pathways responsible for ATP-induced Ca^{2+} influx, we next analysed the role of each of these in ATP-induced Ca^{2+} signalling. First, we examined the contribution of Ca^{2+} influx *via* SOC to the ATP-induced Ca^{2+} signal. When the effect of SK&F96365, a SOC inhibitor, on the repetitive ATP stimulation-induced $[Ca^{2+}]_i$ increase was tested, identical results were seen before and after SK&F96365 exposure (Figure 3C). To further characterize the contribution of SOC to the Ca^{2+} influx, we examined the effect of thapsigargin on the $[Ca^{2+}]_i$ increase. Thapsigargin blocks Ca^{2+} loading of the intracellular Ca^{2+} stores by inhibiting the endoplasmic reticulum Ca^{2+} pump and depleting intracellular Ca^{2+} stores in the absence of receptor activation; this depletion of Ca^{2+} stores then activates SOC. As shown in Figure 4A, the thapsigargin-induced $[Ca^{2+}]_i$ increase in cells in Ca^{2+}-free buffer (trace b) was as great as that in cells in Ca^{2+}-containing buffer (trace a), the mean values for the net thapsigargin-induced $[Ca^{2+}]_i$ changes being 444 ± 60 nM ($n=36$) and 476 ± 63 nM ($n=36$), respectively. These results indicate that depletion of Ca^{2+} stores alone appears insufficient to alter the Ca^{2+} permeability of the plasma membrane, and this is further supported by the results shown in Figures 4B and 5.

In the experiment shown in Figure 4B, we used Mn^{2+}, which presumably enters cells *via* the same pathways as Ca^{2+}, as a substitute for Ca^{2+} in Ca^{2+} influx studies since it cannot be removed from the cytosol by Ca^{2+} transporters, including Ca^{2+} pumps and the Na^+/Ca^{2+} exchanger, and the fluorescence quenching due to Mn^{2+} influx at the isosbestic wavelength can therefore be used as an index of Ca^{2+} influx (Mertz *et al*., 1990). In porcine aortic smooth muscle cells, no difference was seen in the fluorescence quenching induced by Mn^{2+} alone or Mn^{2+} plus thapsigargin (Figure 4B, traces a and b). However, when Mn^{2+} and ATP were added simultaneously, fluorescence quenching was significantly

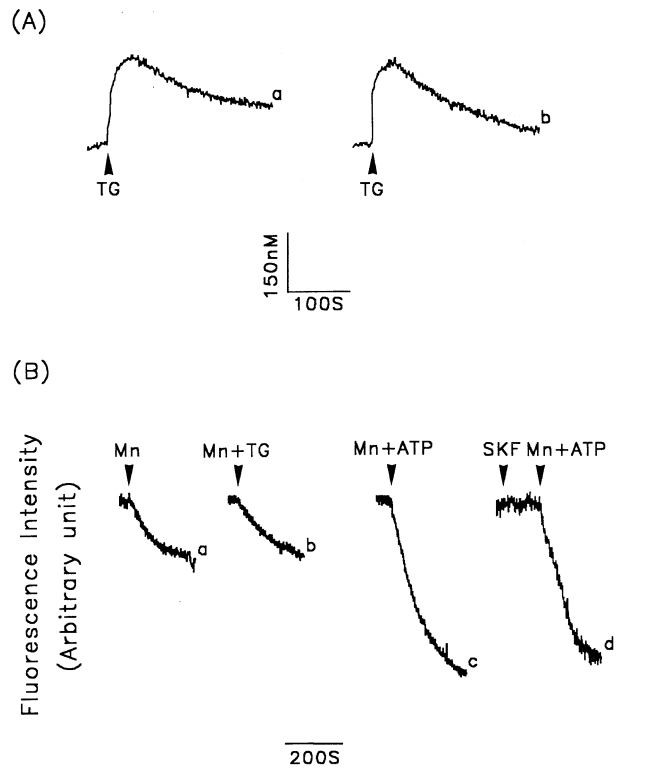

Figure 4 Contribution of the SOC to the thapsigargin-induced $[Ca^{2+}]_i$ increase or the thapsigargin- or ATP-induced Mn^{2+} influx. (A) Thapsigargin (1 μM) was added as indicated (arrowheads) and $[Ca^{2+}]_i$ changes were measured in fura-2-loaded cells in the presence (trace a) or absence (trace b) of extracellular Ca^{2+}. (B) Fura-2-loaded cells were bathed in loading buffer containing 1 mM Ca^{2+}, then 1 mM Mn^{2+} alone (Mn) (trace a), 1 mM Mn^{2+} plus 1 μM thapsigargin (Mn+TG) (trace b), or 1 mM Mn^{2+} plus 30 μM ATP (Mn+ATP (traces c and d) was added, and the fluorescence quenching caused by the Mn^{2+} influx measured. In trace d, the cells were treated with 30 μM SK&F96365 (SKF) for 2 min before the addition of Mn^{2+} plus ATP. Experiments were repeated 36–46 times with similar results; one representative example is shown.

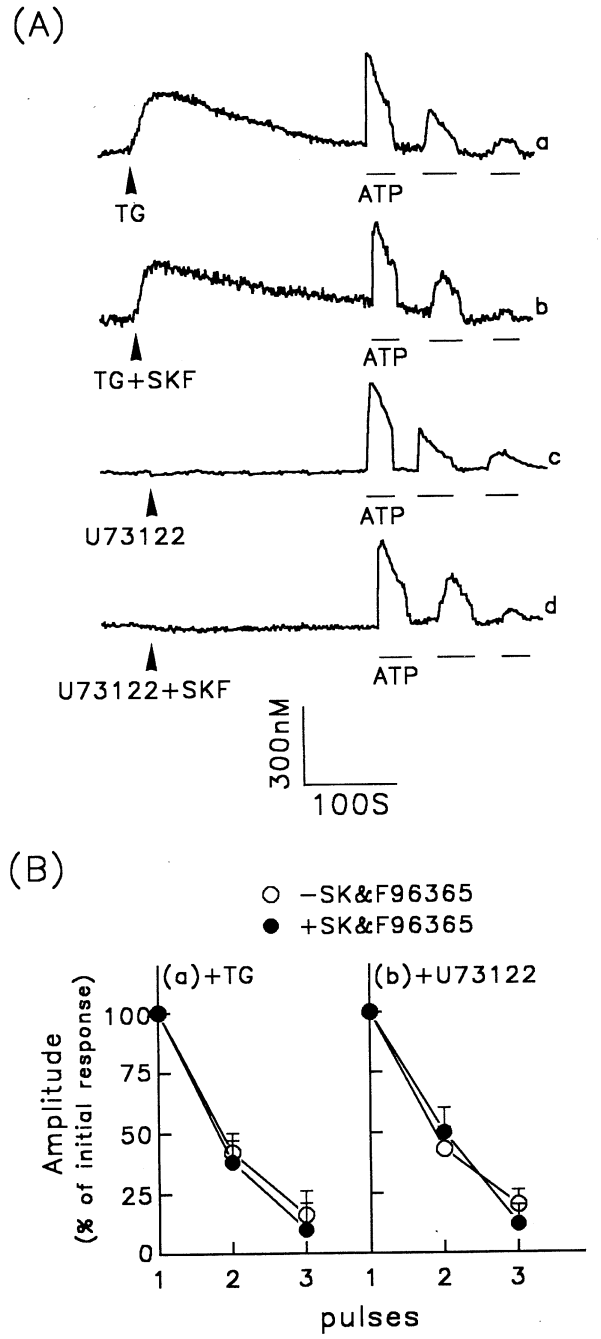

Figure 5 ATP-induced $[Ca^{2+}]_i$ increase *via* Ca^{2+} influx. (A) Five min after the cells were treated with 1 μM thapsigargin (TG) (traces a and b) or 10 μM U-73122 (traces c and d), repetitive ATP-induced $[Ca^{2+}]_i$ increases were measured. In some experiments, 30 μM SK&F96365 was added at the same time as the thapsigargin (TG+SKF) (trace b) or U-73122 (U73122+SKF) (trace d). ATP (30 μM) was applied as indicated by the horizontal bars. (B) Summarized data for the $[Ca^{2+}]_i$ responses to repetitive ATP stimulation following pretreatment of cells with thapsigargin panel (a) or U-73122 panel (b) in the presence or absence of SK&F96365. The amplitudes of the $[Ca^{2+}]_i$ changes are expressed as the peak height for response *n* relative to that for the initial response. The data are the mean ± s.d. for 44–58 experiments.

increased (Figure 4B, trace c), and this effect was not blocked by SK&F96365 (Figure 4B, trace d). Taken together, our results indicate a negligible contribution of SOC to the ATP-induced $[Ca^{2+}]_i$ increase.

We then examined the contribution of other Ca^{2+} influx mechanisms to the ATP-induced Ca^{2+} signal. To block intracellular Ca^{2+} release and SOC activity, we used two different approaches. In addition to depleting the intracellular Ca^{2+} stores using thapsigargin, we also used the phospholipase C inhibitor, U-73122, to block IP_3 generation. As shown in Figure 5A (trace a), following a thapsigargin-induced $[Ca^{2+}]_i$ increase, the $[Ca^{2+}]_i$ declined to a new steady state at which exposure of the cells to ATP still induced an increase in $[Ca^{2+}]_i$, but this was less marked than in control cells not pretreated with thapsigargin (Figure 2A, trace a). Furthermore, the $[Ca^{2+}]_i$ increase was significantly reduced in response to a second exposure to ATP and was only marginal in response to a third exposure. Similar results were seen after treatment with 10 μM U-73122 (Figure 5A, trace c). We have previously shown that U-73122, at a concentration of 10 μM, completely blocks ATP- or SPC-induced IP_3 generation in porcine aortic smooth muscle cells (Chin & Chueh, 1998). SK&F96365 had no effect on the ATP-induced Ca^{2+} influxes after treatment with thapsigargin or U-73122 (Figure 5A, traces b and d). The data for the $[Ca^{2+}]_i$ changes induced by repetitive ATP stimulation under the above conditions are summarized in Figure 5B. Under identical experimental conditions, bradykinin did not induce any $[Ca^{2+}]_i$ increase in cells pretreated with either thapsigargin or U-73122 (data not shown). These results indicate that following pretreatment of cells with concentrations of U-73122 or thapsigargin at which IP_3 generation (U-73122) or Ca^{2+} refilling of, and Ca^{2+} release from, intracellular

Ca^{2+} stores (thapsigargin) are efficiently inhibited, the ATP-induced Ca^{2+} influx desensitized on repeated stimulation.

We next determined the subtype of ATP-gated ion channels involved in the ATP-induced Ca^{2+} influx. As shown in Figure 6A, of the P_{2x} agonists tested (2-MeSATP, AMPCPP, ATPγS, and BzATP), only 2-MeSATP was able to induce a $[Ca^{2+}]_i$ increase; similar results were seen for induction of Mn^{2+} influx (data not shown). 2-MeSATP also acts as a P_{2y} agonist in stimulating Ca^{2+} release *via* phospholipase C activation. To determine the contribution of the 2-MeSATP-mediated Ca^{2+} influx, the 2-MeSATP-induced $[Ca^{2+}]_i$ increase was measured in thapsigargin-treated cells. As shown in Figure 6B, following thapsigargin pretreatment, the $[Ca^{2+}]_i$ increase induced by a second and third exposure to 2-MeSATP was significantly reduced compared to that caused by the initial exposure. The 2-MeSATP-induced $[Ca^{2+}]_i$ increase occurred *via* P_{2x} receptor activation, since it was completely inhibited by pretreatment of cells with 30 μM PPADS for 30 min (Figure 6C).

It was possible that VOC might contribute to the ATP-induced Ca^{2+} signal, since Na^+ influx *via* ATP-gated ion channels (2-MeSATP-sensitive P_{2x} receptors, as described above) can depolarize cells. Furthermore, activation of the P_2 purinoceptor stimulates phospholipase A_2 to produce arachidonic acid (Winitz *et al*., 1994), which can then cause a Ca^{2+} influx, as seen in duckling nasal gland cells (Shuttleworth, 1996). We therefore examined the effects of inhibitors of VOC or phospholipase A_2 on the effects of repetitive ATP stimulation. As shown in Figure 7A, neither a combination of VOC inhibitors (30 μM verapamil, 30 μM nifedipine, and 10 μM ω-conotoxin) (trace a) nor a phospholipase A_2 inhibitor (3 μM arachidonyl trifluoromethyl ketone; $AACOCF_3$) (trace b) had any effect on the repetitive ATP stimulation-induced $[Ca^{2+}]_i$ increases. The data for these changes are summarized in Figure 7B.

We then characterized the toxin sensitivity of the G protein involved in the ATP-induced $[Ca^{2+}]_i$ increase. As shown in Figure 7C (trace a), after 16 h pretreatment of cells with

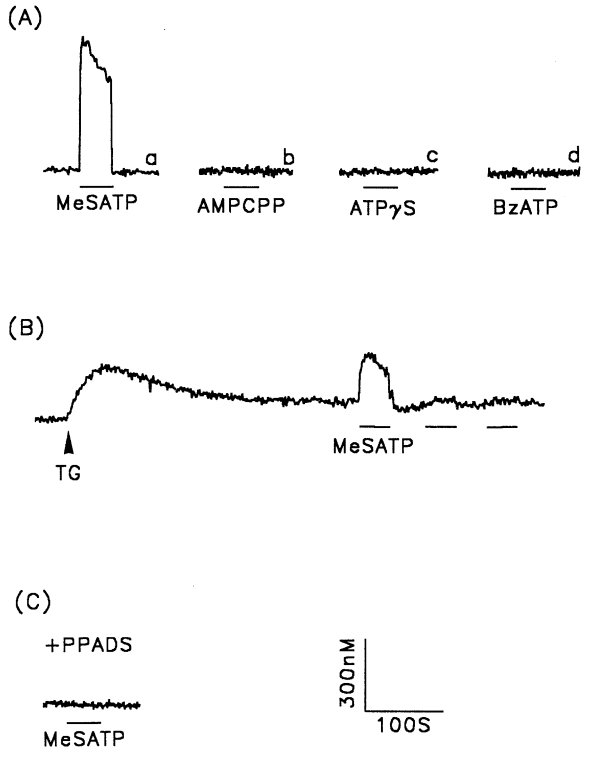

Figure 6 The $[Ca^{2+}]_i$ increase induced by P_{2x} purinoceptor agonists. (A) $[Ca^{2+}]_i$ changes in response to 2-MeSATP (trace a), AMPCPP (trace b), ATPγS (trace c) and BzATP (trace d) (all 30 μM). (B) Five min after the cells were exposed to 1 μM thapsigargin (TG), responses to repetitive 2-MeSATP stimulation, indicated by the horizontal bars, were measured. (C) After 30 min pretreatment of the cells with 30 μM PPADS, 2-MeSATP-induced $[Ca^{2+}]_i$ increases were measured. The concentration of 2-MeSATP was 30 μM in both B and C. Experiments were repeated 26–33 times with similar results; one representative example is shown.

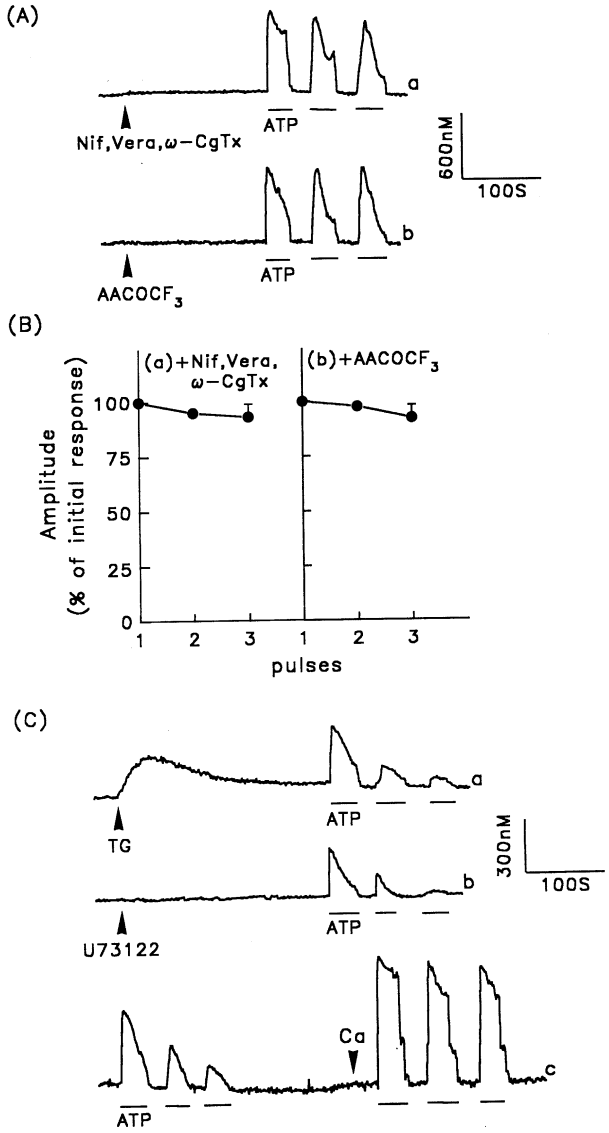

Figure 7 Effect of pertussis toxin, VOC blockers, and a phospholipase A_2 inhibitor on the ATP-induced $[Ca^{2+}]_i$ increases. (A) After treating the cells for 5 min with 30 μM nifedipine, 30 μM verapamil, and 10 μM ω-conotoxin (Nif, Vera, ω-CgTx) (trace a) or 3 μM arachidonyl trifluoromethyl ketone ($AACOCF_3$) (trace b), repetitive ATP-induced $[Ca^{2+}]_i$ increases, indicated by the horizontal bars, were measured in the presence of extracellular Ca^{2+}. (B) Summarized data for the $[Ca^{2+}]_i$ responses following pretreatment of cells with nifedipine, verapamil and ω-conotoxin (panel a) or $AACOCF_3$ (panel b). The data are the mean ± s.d. for 42–56 cells. (C) Cells were pretreated with 100 ng ml^{-1} of pertussis toxin for 16 h, then repetitive ATP-induced $[Ca^{2+}]_i$ increases were measured 5 min after exposure of the cells to 1 μM thapsigargin (TG) (trace a) or 10 μM U-73122 (trace b). Repetitive ATP-induced $[Ca^{2+}]_i$ increases were also measured in the absence of extracellular Ca^{2+} (trace c), and after extracellular Ca^{2+} (2.2 mM) was added back (indicated by the arrowhead). The experiments were repeated 22 times with similar results.

100 ng ml^{-1} of pertussis toxin, neither the thapsigargin-induced $[Ca^{2+}]_i$ increase nor the subsequent repetitive ATP stimulation-induced $[Ca^{2+}]_i$ increases differed significantly from those in control cells (compare with Figure 5A, trace a). Similar results were seen when U-73122 was used instead of thapsigargin (Figure 7C, trace b). In the absence of extracellular Ca^{2+}, the ATP-induced $[Ca^{2+}]_i$ increase presumably occurs *via* the phospholipase C signalling cascade, which is pertussis toxin-insensitive, and when Ca^{2+} was reintroduced into the bathing solution, a reproducible $[Ca^{2+}]_i$ increase profile was still seen in response to repetitive ATP stimulations (Figure 7C, trace c). A similar lack of sensitivity was seen when cells were treated with 1 μg ml^{-1} of cholera toxin for 16 h (data not shown).

SPC, the enzymic product of sphingomyelin deacylase, has been shown to act as an extracellular signal (Meyer zu Heringdorf *et al*., 1997). In porcine aortic smooth muscle cells, it induces an increase in $[Ca^{2+}]_i$ predominantly *via* IP_3-sensitive Ca^{2+} release (Chin & Chueh, 1998). We therefore measured its effect on the $[Ca^{2+}]_i$ increases seen on repetitive stimulation. As shown in Figure 8, the $[Ca^{2+}]_i$ increases gradually decreased and were hardly detectable on the third exposure both in the presence or absence of extracellular Ca^{2+} (Figure 8A and B, trace a). In contrast to the response to ATP (Figure 3B), the desensitized response to SPC, seen in the absence of extracellular Ca^{2+}, was not restored by re-addition of Ca^{2+} to the bathing buffer (Figure 8B, trace a), but was restored by pretreatment of cells with staurosporine for 5 min (Figure 8A and B, trace b). Since staurosporine was a rather nonspecific kinase inhibitor inhibiting protein kinases A, C and G, we next examined the effect of Rp-cAMPS, Gö 6983, KT5823 and KN-62, the specific inhibitor for protein kinase A, C and G and CaM kinase II, respectively, on repetitive SPC stimulation-induced $[Ca^{2+}]_i$ increase. Of the inhibitors tested, only Gö 6983 was able to restore the desensitized Ca^{2+} response to SPC (Figure 8A, traces c–f); similar results were seen in the absence of extracellular Ca^{2+} after Ca^{2+} was added back to the bathing buffer (Figure 8B, traces c–f).

Our results indicate that, in addition to IP_3 generation, ATP also stimulated Ca^{2+} influx *via* a 2-MeSATP-sensitive P_{2x} receptor, leading to a $[Ca^{2+}]_i$ increase. Thus, although both ATP and SPC activated phospholipase C, the action of SPC could be desensitized, while that of ATP could not. However, desensitization of the repetitive SPC-induced $[Ca^{2+}]_i$ increase could be reversed by inhibition of protein kinase C-induced protein phosphorylation. To characterize the underlying mechanism, we next measured ATP- or SPC-induced IP_3 generation, with or without prior treatment of the cells with staurosporine. As shown in Figure 9A, in control cells without staurosporine pretreatment, ATP-induced IP_3 generation reached a peak of 20.3 ± 1.9 ($n=6$) pmol mg^{-1} protein after 15 s, then fell to a plateau where it remained for at least 4 min, while the peak level after SPC treatment was significantly less, reaching a peak of 6.3 ± 0.9 ($n=6$) pmol mg^{-1} protein in 15 s, and returning to the basal level within 60 s. Following pretreatment of cells with staurosporine, basal and ATP- or SPC-stimulated IP_3 generation was similar to that seen in control cells (Figure 9B). Thus, ATP- or SPC-induced IP_3

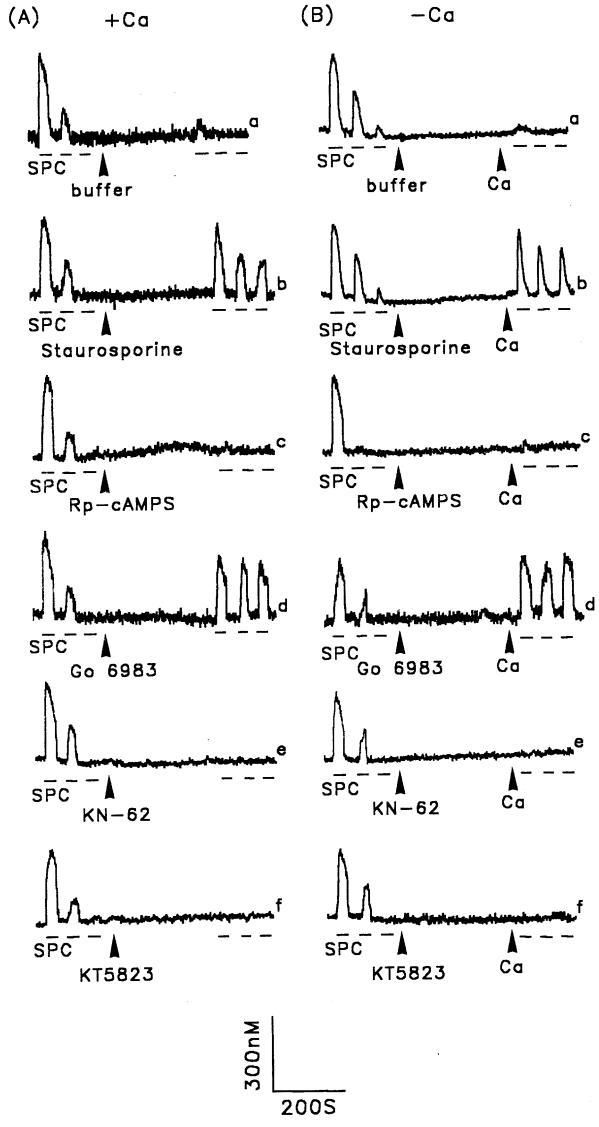

Figure 8 Effect of kinase inhibitors on SPC-induced $[Ca^{2+}]_i$ increases in the presence or absence of extracellular Ca^{2+}. Repetitive SPC-induced $[Ca^{2+}]_i$ increases to 5 μM SPC were initially measured in the presence (A) or absence (B) of extracellular Ca^{2+}, then after the cells had been treated with buffer (A and B, trace a), 0.1 μM staurosporine (A and B, trace b), 30 μM Rp-cAMPS (A and B, trace c), 0.3 μM Gö 6983 (A and B, trace d), 10 μM KN-62 (A and B, trace e) or 1 μM KT5823 (A and B, trace f), or after adding back 2.2 mM Ca^{2+} to the bathing solution (B, all traces), as indicated by the arrowheads. Experiments were repeated 32–51 times with similar results; one representative example is shown.

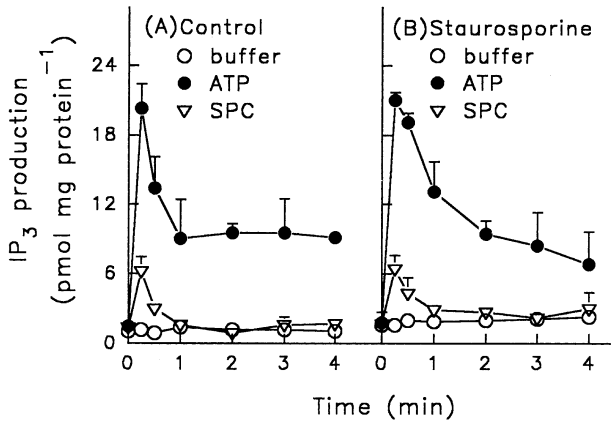

Figure 9 Effect of staurosporine on ATP- or SPC-induced IP_3 generation in porcine aortic smooth muscle cells. Control cells (A) or cells pretreated with 0.1 μM staurosporine for 5 min (B) were stimulated with buffer, 30 μM ATP, or 5 μM SPC for the indicated time periods, then the IP_3 was extracted and measured using a radioreceptor assay. The data are means ± s.d. for six independent experiments.

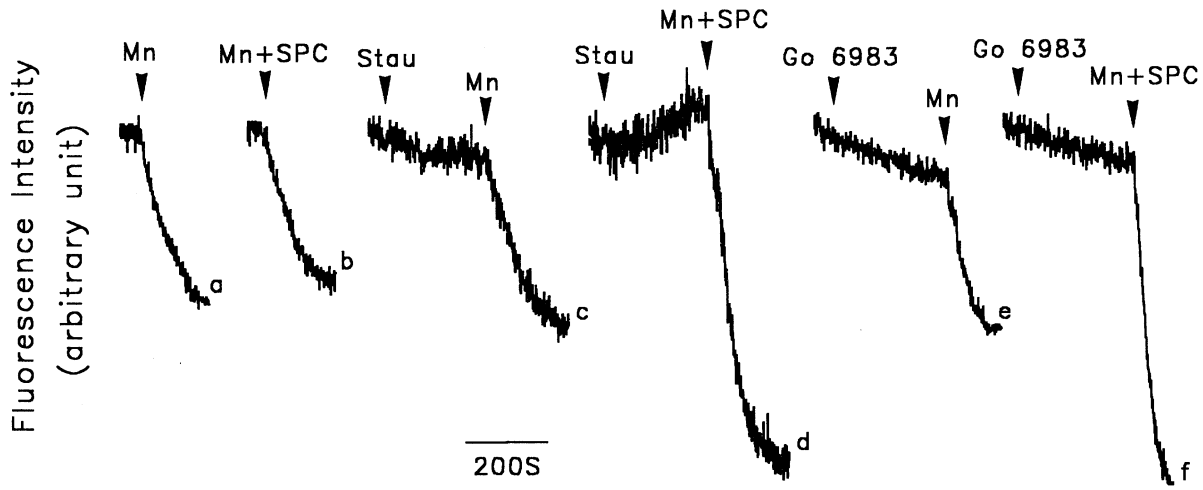

Figure 10 Effect of kinase inhibitors on the SPC-induced Mn^{2+} influx in porcine aortic smooth muscle cells. Fura-2-loaded cells were bathed in loading buffer containing 1 mM Ca^{2+}. The cells were then treated for 5 min with buffer (traces a and b), 0.1 μM staurosporine (traces c and d) or 0.3 μM Gö 6983 (traces e and f). 1 mM Mn^{2+} (Mn) (traces a, c and e) or 1 mM Mn^{2+} plus 5 μM SPC (Mn + SPC) (traces b, d and f) was then added and the fluorescence quenching due to Mn^{2+} influx measured. The experiments were repeated 28–36 times with similar results; one representative result is shown.

generation in control cells did not differ significantly from that in staurosporine-treated cells. In porcine aortic smooth muscle cells, Ca^{2+} release from intracellular Ca^{2+} stores was predominantly attributable to the action of IP_3, since neither caffeine (20 mM) nor ryanodine (10 μM) induced $[Ca^{2+}]_i$ increase (data not shown).

We finally examined whether protein kinase C inhibitor increased the Ca^{2+} permeability in SPC-stimulated cells, using Mn^{2+} influx as an indicator of Ca^{2+} influx. As shown in Figure 10, SPC did not stimulate Mn^{2+} influx, since the fluorescence quenching was indistinguishable from that in control cells (traces a and b). The same was true after cells have been pretreated with Rp-cAMPS, KT5823 or KN-62 (data not shown). However, following pretreatment of cells with staurosporine or Gö 6983, the fluorescence quenching induced by SPC was significantly increased (traces c and d, and traces e and f, respectively)

Discussion

Our results show that, in porcine aortic smooth muscle cells, ATP induces both intracellular Ca^{2+} release and Ca^{2+} influx. Firstly, the ATP-induced $[Ca^{2+}]_i$ increase was greater, and the time required for the $[Ca^{2+}]_i$ to fall to the basal level longer, in Ca^{2+}-containing than in Ca^{2+}-free buffer (Figure 1). Secondly, the ATP-induced $[Ca^{2+}]_i$ increase was still seen after cells were pretreated with U-73122 or thapsigargin (Figure 5), indicating that Ca^{2+} influx is activated by ATP, since U-73122 inhibits phospholipase C (Smallridge *et al*., 1992), while thapsigargin depletes the intracellular Ca^{2+} stores (Thastrup *et al*., 1990). Finally, the Mn^{2+} influx induced by ATP is further support for an ATP-induced Ca^{2+} influx. In general, Ca^{2+} influxes are mediated by activation of ROC, VOC and SOC. We further demonstrated that the $P2X_5$ purinoceptor (ROC) was responsible for the ATP-induced Ca^{2+} influx, and that VOC and SOC were not involved (see below).

In many nonexcitable cells, capacitative Ca^{2+} entry *via* SOC can be stimulated by store depletion (Luckhoff & Clapham, 1992; Hoth & Penner, 1992). The results of this study showed SOC activity (trp channels) to be negligible in porcine aortic smooth cells. Firstly, not only was the Mn^{2+} influx unchanged from the basal level in the presence of thapsigargin (Figure 4B), but the thapsigargin-induced $[Ca^{2+}]_i$ increases were identical in the presence or absence of extracellular Ca^{2+} (Figure 4A). If depletion of the Ca^{2+} stores altered the permeability of the plasma membrane to Ca^{2+} *via* activation of SOC, a higher $[Ca^{2+}]_i$ increase in the presence of extracellular Ca^{2+} or a greater Mn^{2+} influx in response to thapsigargin would be expected. The ineffectiveness of thapsigargin in inducing Mn^{2+} or Ca^{2+} influx in the current study is consistent with a recent study in human embryonic kidney 293 cells transfected with the human Trp3 (hTrp3) gene, in which expression of the hTrp3 gene formed a nonselective cation channel that opened after activation of phospholipase C, but not after store depletion (Zhu *et al*., 1998). Secondly, both the ATP-induced $[Ca^{2+}]_i$ increase and the Mn^{2+} influx were not affected by the SOC inhibitor, SK&F96365 (Figures 3C and 4B), ruling out involvement of SOC in the ATP-induced Ca^{2+} influx. Thirdly, pretreatment of cells with U-73122 or thapsigargin inhibits the phospholipase C signalling cascade; U-73122 inhibits phospholipase C, while thapsigargin depletes the intracellular Ca^{2+} stores and may cause SOC-induced Ca^{2+} influx. Thus, the ATP-induced $[Ca^{2+}]_i$ increases seen in the presence of U-73122 or thapsigargin reflect, respectively, the activity of VOC and ROC, or VOC, ROC and SOC. The amplitudes of the $[Ca^{2+}]_i$ increases on multiple stimulation were identical in the presence of these two inhibitors and were not affected by the SOC inhibitor, SK&F96365 (Figure 5), suggesting that, in porcine aortic smooth muscle cells, the contribution of SOC to the ATP-induced Ca^{2+} influx is negligible. Thus, the possible mechanisms responsible for the ATP-induced Ca^{2+} influx are ROC and VOC.

According to the classical classification, both P_{2x} and P_{2z} purinoceptors (ROC) may be involved in the ATP-induced Ca^{2+} influx. The fact that BzATP, a P_{2z} ($P2X_7$) purinoceptor-selective agonist, could not induce a $[Ca^{2+}]_i$ increase or Mn^{2+} influx ruled out involvement of the P_{2z} purinoceptor in porcine aortic smooth muscle cells. The effectiveness of 2-MeSATP, the ineffectiveness of AMPCPP and ATPγS, and the blockade of the $[Ca^{2+}]_i$ increase by 30 μM PPADS further suggest that activation of the $P2X_5$ purinoceptor is responsible for the ATP-induced Ca^{2+} influx (Figure 6) (North & Barnard, 1997; Kunapuli & Daniel, 1998; Boarder & Hourani, 1998). Opening

of ROC allows a Na^+ influx, which may subsequently depolarize the membrane potential and cause a VOC-mediated Ca^{2+} influx. Indeed, in pituitary gonadotrophs, the ATP-induced $[Ca^{2+}]_i$ response is partially inhibited by nifedipine (Tomic *et al*., 1996). Similar results are seen in NG108-15 cells, in which the ATP-induced Ca^{2+} influx *via* P_{2x} or P_{2z} is inhibited approximately 34–76% by VOC blockers, including ω-conotoxin, nifedipine, and verapamil (Chueh & Kao, 1993). In the present study, the insensitivity of the ATP-induced $[Ca^{2+}]_i$ increase to nifedipine, verapamil, and ω-conotoxin suggests that, in porcine aortic smooth muscle cells, VOC is not involved (Figure 7).

It is possible that the ATP-induced $[Ca^{2+}]_i$ increase may be partially due to the release of arachidonic acid, since phospholipase A_2 is reported to be stimulated by ATP (Winitz *et al*., 1994) and arachidonic acid mobilizes Ca^{2+} by either activating Ca^{2+} influx or triggering Ca^{2+} release (Vacher *et al*., 1989). A recent study has also shown that depletion of Ca^{2+} stores by arginine vasopressin or thapsigargin can induce arachidonic acid release in A-10 smooth muscle cells (Wolf *et al*., 1997). In the present study, the ATP-induced $[Ca^{2+}]_i$ increase was not blocked by a phospholipase A_2 inhibitor, suggesting that arachidonic acid is not involved in this process in porcine aortic smooth muscle cells.

The inability of caffeine or ryanodine to produce a $[Ca^{2+}]_i$ increase suggests that Ca^{2+} release from the intracellular Ca^{2+} stores is mainly mediated by activation of the IP_3 receptor, rather than by Ca^{2+}-induced Ca^{2+} release. The reduced responses seen on repetitive ATP stimulation in the absence of extracellular Ca^{2+} suggest either that the receptor-G protein-phospholipase C coupling system desensitizes or that Ca^{2+} contained in the intracellular Ca^{2+} stores is not available after several stimulations due to store depletion (Figure 2). Indeed, the IP_3 generation induced by ATP or SPC, even in the presence of extracellular Ca^{2+}, was transient, with a rapidly declining peak (Figure 9) and, in the absence of extracellular Ca^{2+}, the fullness of the Ca^{2+} stores was significantly reduced after repetitive ATP stimulation (Figure 3). The facts that Ca^{2+} levels in the intracellular Ca^{2+} stores were not reduced following multiple stimulations in the presence of extracellular Ca^{2+} (Figure 3) and the ATP responses fully recovered on reintroduction of Ca^{2+} suggest that to have reproducible ATP effects on the $[Ca^{2+}]_i$ increase the filling state of the intracellular Ca^{2+} stores is critical. The decrease in the ATP responses following thapsigargin or U-73122 pretreatment (Figure 5) suggests that the ATP-induced Ca^{2+} influx desensitizes. It appears that, under physiological conditions, following repetitive ATP stimulation, even the reduced Ca^{2+} influx seen in a desensitized state is sufficient to refill the partially depleted intracellular Ca^{2+} stores and leads to a reproducible $[Ca^{2+}]_i$ increase. The recovery of the SPC-induced Ca^{2+} response due to inhibition of protein kinase C-induced phosphorylation is mainly attributable to Ca^{2+} influx, rather than to IP_3 generation (Figures 9 and 10), further indicating the pivotal role of the intracellular Ca^{2+} stores in the Ca^{2+} signalling pathway.

In conclusion, we have demonstrated that, in porcine aortic smooth muscle cells, activation of P_2 purinoceptors by ATP induces an increase in $[Ca^{2+}]_i$ which is insensitive to pertussis toxin and cholera toxin. In addition to triggering intracellular Ca^{2+} release *via* phospholipase C activation, ATP also stimulates Ca^{2+} influx *via* $P2X_5$ receptors. Although the activity of $P2X_5$ receptor progressively decreases following repetitive stimulation with ATP, the overall Ca^{2+} signal induced by ATP remains the same if the releasing and refilling pathways of the intracellular Ca^{2+} stores are not inhibited, suggesting involvement of the intracellular Ca^{2+} stores in the Ca^{2+} signalling pathway. Our data further show that depletion of the intracellular Ca^{2+} stores alone does not alter the plasma membrane permeability to Ca^{2+}. In contrast, the SPC-activated Ca^{2+} signalling pathway is solely mediated by the phospholipas C cascade, is distinct from that activated by ATP, and desensitizes. The desensitization of the SPC-induced Ca^{2+} signal can be reversed by inhibition of protein kinase C, which stimulates Ca^{2+} influx, further supporting the importance of the refilling of intracellular Ca^{2+} stores by influxed Ca^{2+} in the Ca^{2+} signalling pathway.

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