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Extracellular ATP triggers two functionally distinct calcium signalling pathways in PC12 cells

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SUMMARY

We have investigated the effects of extracellular ATP on Ca^{2+} signalling, and its relationship to secretion in rat pheochromocytoma (PC12) cells. In single cells, extracellular ATP evoked two very distinct subcellular distributions of intracellular calcium concentration ($[Ca^{2+}]_i$), only one of which could be mimicked by the pyrimidine nucleotide UTP, suggesting the involvement of more than one cell surface receptor in mediating the ATP-induced responses. ATP and UTP were equipotent in activating a receptor leading to inositol phosphate production and the mobilisation of intracellular Ca^{2+}. In some cells (19%) this rise in $[Ca^{2+}]_i$ initiated at a discrete site and then propagated across the cell in the form of a Ca^{2+} wave. In addition to mobilising intracellular Ca^{2+} through a 'nucleotide' receptor sensitive to ATP and UTP, the results indicate that ATP also activates divalent cation entry through an independent receptor-operated channel. Firstly, ATP-induced entry of Ca^{2+} or Mn^{2+} was independent of Ca^{2+} mobilisation, as prior treatment of cell populations with UTP abolished the ATP-evoked release of intracellular Ca^{2+} stores, but left the Ca^{2+}- and Mn^{2+}-entry components uneffected. Secondly, although UTP and ATP were equally effective in generating inositol phosphates, only ATP stimulated divalent cation entry, indicating that ATP-activated influx was independent of phosphoinositide turnover. Thirdly, single cell experiments revealed a subpopulation of cells that responded to ATP with divalent cation entry without mobilising Ca^{2+} from intracellular stores. Lastly, the dihydropyridine antagonist, nifedipine, reduced the ATP-induced rise in $[Ca^{2+}]_i$ by only 24%, suggesting that Ca^{2+} entry was largely independent of L-type voltage-operated Ca^{2+} channels. The Ca^{2+} signals could also be distinguished at a functional level. Activation of ATP-induced divalent cation influx was absolutely required to evoke transmitter release, because ATP triggered secretion of $[^3H]$dopamine only in the presence of external Ca^{2+}, and UTP was unable to promote secretion, irrespective of the extracellular $[Ca^{2+}]$. The results suggest that the same extracellular stimulus can deliver different Ca^{2+} signals into the same cell by activating different Ca^{2+} signalling pathways, and that these Ca^{2+} signals can be functionally distinct.

Key words: calcium, fura-2, exocytosis, ATP receptor, PC12 cell

INTRODUCTION

It is becoming increasingly apparent that extracellular ATP plays an important role in cellular signalling. ATP has been shown to mediate a range of physiological effects in different cell types, such as vasoconstriction, ion flux and secretion (Kennedy and Burnstock, 1985; Soltoff et al., 1990; Cockcroft and Gomperts, 1980) and there is much evidence to suggest a role for ATP as a neuromodulator in both the peripheral and central nervous systems (CNS) (Silinsky and Ginsborg, 1983; Charest et al., 1985). Additionally, ATP has been shown to mediate synaptic currents, suggesting that ATP acts as a fast neurotransmitter in the CNS (Benham, 1992; Edwards et al., 1992).

Extracellular ATP activates a group of cell surface receptors classed as purinergic. P_1 purinoceptors are sensitive to adenosine, whereas P_2 purinoceptors are activated predominantly by ATP and its close analogues (Burnstock, 1978). Activation of ATP-sensitive receptors triggers phosphoinositide metabolism in hepatocytes and aortic endothelial cells (Charest et al., 1985; Hallam and Pearson, 1986), mobilisation of intracellular Ca^{2+} stores in chromaffin cells and pancreatic islets (Kim and Westhead, 1989; Sasakawa et al., 1989; Geshwind et al., 1989), influx of external Ca^{2+} in pancreatic HIT cells and rabbit ear artery smooth muscle (Geshwind et al., 1989; Benham and Tsien, 1987), and stimulation of prostanoid production and release in primary astrocytes (Pearce et al., 1989).

The secretory granules of the rat pheochromocytoma (PC12) cell, like their non-tumorigenic counterpart the adrenal chromaffin cell, contain ATP:catecholamine in a ratio of 1:4 (Phillips, 1982). Therefore, on granule release the concentration of extracellular ATP could be envisaged to reach concentrations high enough to interact with purinergic receptors and modulate transmitter release in response to other secretory

stimuli. In order to gain more insight into the relationship between activation of ATP receptors, the production of intracellular messengers and the modulation of secretion, we have studied the relationship between ATP receptor activation and triggering of Ca^{2+}-dependent catecholamine secretion from PC12 cells. The results indicate that extracellular ATP can elevate the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) by activating two independent signalling pathways, and that only one of these Ca^{2+} signals is capable of triggering a secretory response.

MATERIALS AND METHODS

Materials

Unless otherwise stated, all chemicals were obtained from Sigma (St Louis, MO). Culture materials from GIBCO Ltd. (Paisley, Scotland).

Methods

Cell culture

PC12 cells were obtained from the European Cell Culture Laboratories (Porton Down, UK). Cells were grown at 37°C, 5% CO_2 on 10 cm plastic dishes coated with 1 mg/ml poly-L-ornithine in 85% RPMI growth medium supplemented with L-glutamine, 10% horse serum, 5% fetal calf serum, 100 i.u./ml of penicillin/streptomycin, 100 μg/ml fungizone, and buffered with 2.2 g/l sodium bicarbonate.

Intracellular Ca^{2+} measurements

Single cells

For digital-video imaging, cells were adhered to poly-L-ornithine-coated 22 mm coverslips. Coverslips were mounted in a metal chamber and incubated for 30 minutes in loading buffer (LB) containing RPMI + L-glutamine, 5% fetal calf serum, 200 μM sulphinpyrazone and 3 μM fura-2-AM, at 37°C. After loading, the cells were washed in fresh buffer consisting of: 145 mM NaCl, 5 mM KCl, 20 mM HEPES, 1 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 10 mM glucose, 3 mM $CaCl_2$ (KR medium) at pH 7.4, and imaged 5-10 minutes later. Cells were imaged after positioning the chamber on the stage of a Nikon Diaphot inverted epi-fluorescent microscope. High-pressure twin xenon arc lamps provided alternative excitation at 340 nm and 380 nm, while emitted light was filtered at 510 nm and collected by an intensified CCD camera (Photonic Science, Robertsbridge, UK). The video-signal from this was digitised and stored in an Imagine image processing system (Synoptics Ltd, Cambridge, UK), hosted by a DEC MicroVAX II computer. The excitation source was switched by a rotating-mirror chopper (Glen Creston Instruments, Stanmore, UK) driven by a stepping motor and synchronised with the video timebase to give alternate TV frames at each of the two wavelengths. The Imagine video-rate processor was programmed to form from each successive pair of frames a 'live' ratio image, which was recursively filtered with a 200 ms time constant (i.e. 5 ratio images/second), and stored on videotape (SonyUmatic) for subsequent processing. Full details of this imaging system are given by Moreton (1991).

Formation of the ratio image was implemented in a look-up table, computed from the formula given by Grynkiewicz et al. (1985):

$$[Ca^{2+}]_i = K_d \frac{R-R_{min}}{R_{max}-R} \frac{S_{f2}}{S_{b2}},$$

where K_d is the dissociation constant for fura-2/Ca^{2+} (224 nM), R is the intensity ratio for fluorescence at the two chosen wavelengths, R_{min} and R_{max} are ratios at zero and saturating $[Ca^{2+}]_i$, respectively, and S_{f2}/S_{b2} is the ratio of excitation efficiencies for free and bound fura-2 at the higher of the two wavelengths. All ratios were determined empirically using the in vitro calibration method (Moreton, 1992) by measuring the fluorescence intensities of bulk solutions of fura-2 free acid in $CaCl_2$/EGTA buffers prepared in an intracellular medium. This calibration method underestimates $[Ca^{2+}]_i$ by 25-30% compared to the in situ calibration method used for cell populations (see below), because the lack of polarity and viscocity in the bulk solutions leads to an overestimate of both R_{min} and R_{max} by about 15% (Moreton, 1992).

Recorded video data were played back through Imagine, using a different programme to give a false-colour representation of image intensities, and to allow individual pictures to be captured on disc. The false-colour images presented depict the ratio image in either resting or ATP-stimulated cells (see Fig. 2) or the ratio image in ATP-stimualted cells in the presence of external Mn^{2+} (see Fig. 9A).

Agonists were applied either via general perfusion or via a U-tube positioned 2-3 mm from the cells under observation, ensuring that the cells come into contact with agonist within a second timescale.

Populations

Cells were harvested from tissue culture dishes when approaching confluence by dissociation in trypsin/EDTA buffer. Cells were washed by centrifugation (1000 ***g***, for 5 minutes) and resuspended at a density of 5×10^6 cells/ml in loading buffer (LB) supplemented with 200 μM sulphinpyrazone and 3 μM fura-2-AM. After 30 minutes at 37°C with constant stirring, cells were washed twice and resuspended at 10×10^6 cells/ml in KR medium supplemented with 200 μM sulphinpyrazone.

Cell suspensions (300 μl) were transferred to a stirred thermostatically (37°C) controlled cuvette. Fluorescence was continuously monitored with a Perkin-Elmer LS-5 luminescence spectrophotometer (alternate excitation wavelengths 340 nm and 380 nm) and the information was stored using Perkin-Elmer Fura-2 analysis software on an IBM personal computer. The traces were calibrated from the equation given by Grynkiewicz et al. (1985):

$$[Ca^{2+}]_i = K_d \times \frac{(F-0.33(F_{max}-F_{mn}))}{(F_{max}-F)}.$$

Calibration was carried out using the in situ method (Moreton, 1992). F_{max} was obtained by adding 50 μM digitonin to permeabilise the cells, and F_{mn} by the addition of 4 mM $MnCl_2$ to quench the fura-2 signal. A K_d of 224 nM (37°C) was used for the fura-2/Ca^{2+} complex (Grynkiewicz et al., 1985). Agonists were pipetted into the cuvette from stock solutions to give the correct final concentration.

Manganese quench

The technique of monitoring Mn^{2+} quench of fura-2 (Hallam and Rink, 1985) was used at both the single cell and cell population levels. Unless otherwise stated 200-300 μM $MnCl_2$ was included in the external medium containing 3 mM $CaCl_2$. Fluorescence was monitored at the isosbestic point of fura-2 free acid, 360 nm. For investigation of the spatial distribution of Mn^{2+}, fura-2-loaded single cells were ratio imaged (340 nm/380 nm) as described by Cheek et al. (1993). This technique allows the visualisation of the Mn^{2+} quench whilst avoiding any spatial inconsistencies arising from uneven intracellular fura-2 distribution.

Inositol phosphate measurements

PC12 cells were incubated for 24 hours in inositol-free RPMI supplemented with 5% dialysed mixed serum, 2.2 g/l sodium bicarbonate and 1 μCi/ml myo-[^{3}H]inositol (86 Ci/mmol, Amersham International). Cells were removed from their dishes by gentle agitation, centrifuged (1000 ***g***, 5 minutes) and resuspended in KR medium at 37°C. After 30 minutes, 30 mM LiCl was added and 10 minutes later the agonist was applied. After a further 10 minute incubation period the reactions were terminated by rapidly heating the cells to ≈90°C

for 1 minute. Cell debris was pelleted by centrifugation at 2000 ***g*** for 10 minutes and the resulting supernatants applied to anion exchange columns (AG1X8, formate form, 200-400 mesh, Bio-Rad). [^{3}H]inositol and [^{3}H]glycerophosphoinositol were eluted with 8 ml of 60 mM ammonium formate/5 mM Na_2BO_4, InsP_1 with 8 ml of 0.2 M ammonium formate/0.1 M formic acid, InsP_2 with 8 ml of 0.5 M ammonium formate/0.1 M formic acid, InsP_3 and InsP_4 together eluted with 8 ml of 1.25 M ammonium formate/0.1 M formic acid. The activity in each fraction was expressed as a fraction of the total label incorporated into the cells.

Secretion assays

Secretion was assayed by measuring the release of [^{3}H]dopamine. Cells were loaded at a density of 1×10^6 cells/ml in culture medium supplemented with 1.25×10^5 dpm/ml of [^{3}H]dopamine and ascorbic acid, and incubated at 37°C, 5% CO_2 for 40 minutes. After washing three times, cells were resuspended at 2.5×10^6 cells/ml in KR medium with or without $CaCl_2$. A 100 μl sample of agonist was added to 100 μl of cell suspension and the mixture incubated for 10 minutes at 37°C. The reaction was terminated by adding 200 μl ice-cold KR medium containing 20 mM EGTA. After centrifugation (11,600 ***g***, 2 minutes) 150 μl of the supernatant was removed for counting in a scintillation counter by adding 1 ml of Ecoscint A scintillation fluid (National Diagnostics). Results were expressed as a fraction of total [^{3}H]dopamine incorporated into the cell (determined by total disruption of the cell membrane using 1% Triton X-100) with basal release assigned as 100%.

Fluorescent antibody studies

Immunofluorescent staining with anti-dopamine-β-hydroxylase (anti-DBH) was carried out essentially as described previously (Cheek et al., 1989). Cells were adhered to 11 mm diameter glass coverslips coated in poly-L-ornithine. Coverslips were washed in KR medium including 0.1% BSA at 37°C and then incubated in agonist made up in KR solution in the presence of anti-DBH × 1/5 (antibody, gift from Prof. R. D. Burgoyne, Secretory Control Group, Department of Physiology, Liverpool University). After 15 minutes of incubation, medium was removed and the cells washed twice in fresh KR. The cells were subsequently fixed in 4% formaldehyde in phosphate buffered solution (PBS) for 2 hours at room temperature. After washing twice, the cells were first incubated in 0.3% bovine serum albumen in PBS to block any non-specific binding sites, and then with anti-rabbit biotin (×1/100). The medium was removed after 60 minutes and replaced with Texas Red-linked streptavidin (×1/50) for a further 30 minutes. Finally, the cells were mounted in PBS on a glass coverslip, sealed and observed under a fluorescence microscope.

RESULTS

Effects of ATP on $[Ca^{2+}]_i$

Intact fura-2-loaded PC12 populations were challenged with extracellular ATP (100 μM) in the presence and absence of external Ca^{2+}. Fig. 1A illustrates that, in Ca^{2+}-free con-

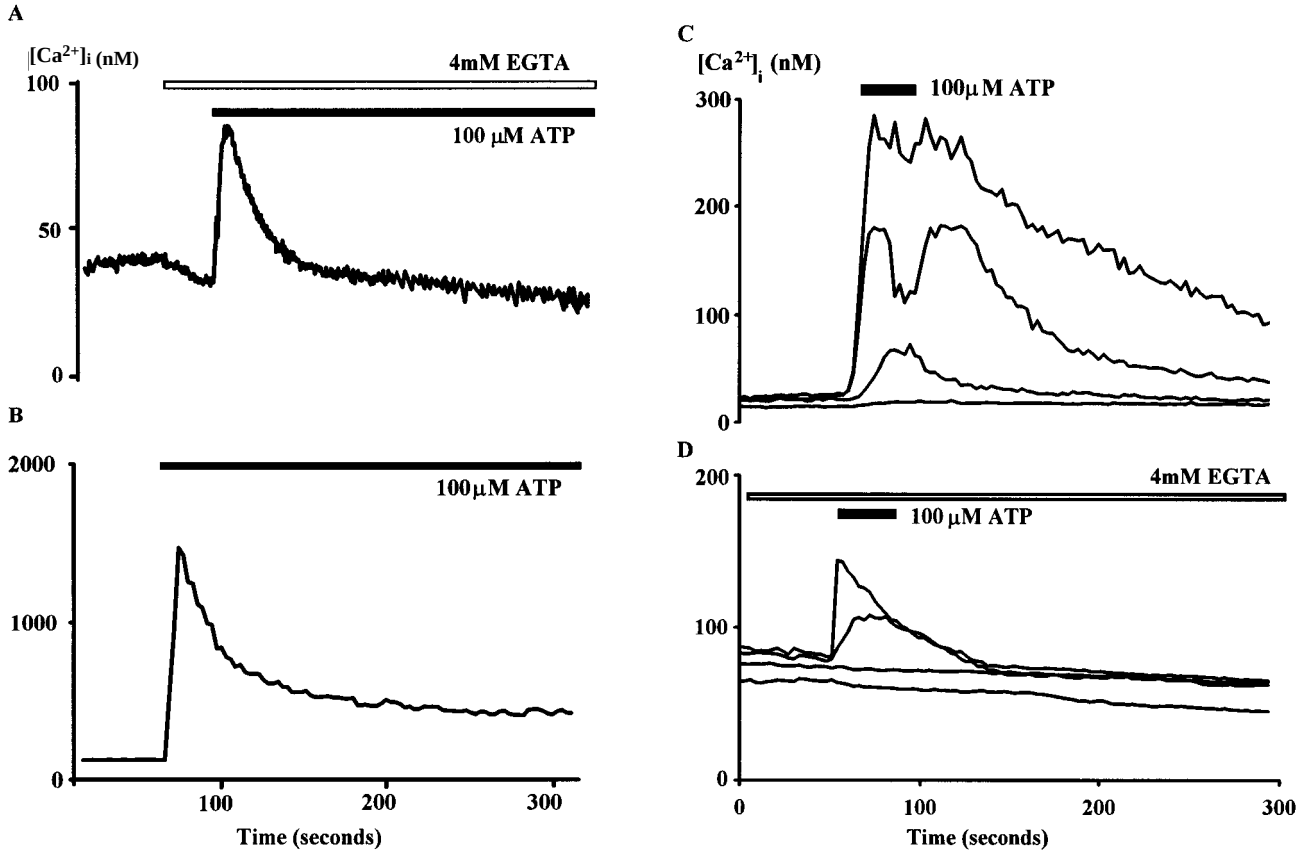

Fig. 1. Traces illustrating the time course of $[Ca^{2+}]_i$ after challenging PC12 cells with extracellular ATP in the presence and absence of external Ca^{2+}. (A) Typical response of a PC12 population to 100 μM ATP in the presence of 1 mM excess EGTA. Under these conditions $[Ca^{2+}]_i$ rose by 74±6.8 nM (n=5). (B) The response to 100 μM ATP in a typical cell population in the presence of 3 mM external Ca^{2+}; ATP increased $[Ca^{2+}]_i$ by 1.2±0.24 μM (n=5). The Ca^{2+} responses of four individual PC12 cells to 100 μM ATP in the presence of 3 mM external Ca^{2+} (C) or 1 mM excess EGTA (D). 112/125 (90%) of single cells responded to 100 μM ATP in Ca^{2+} containing medium; 36/92 (39%) of single cells responded in the absence of external Ca^{2+}.

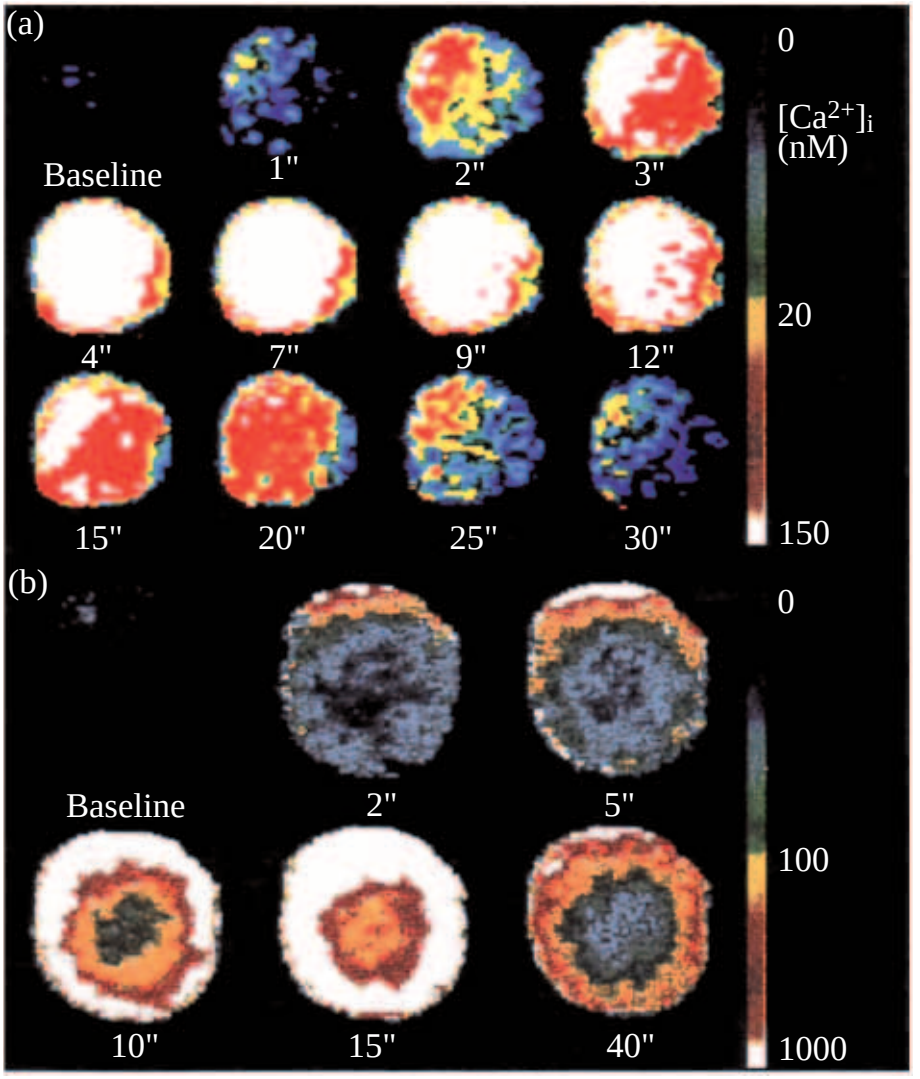

Fig. 2. Single cell montages illustrating the time course of $[Ca^{2+}]_i$ changes in an individual PC12 cell to ATP in the presence and absence of extracellular Ca^{2+}. Single fura-2-loaded PC12 cells were challenged with 100 μM ATP in the presence of 1 mM excess EGTA (a) or in the presence of 3 mM external Ca^{2+} (b). Note that the rise in $[Ca^{2+}]_i$ was larger in the presence of external Ca^{2+}. In the absence of external Ca^{2+}, ATP application resulted in a localised release of Ca^{2+} followed by a propagating wave of Ca^{2+} that migrated across the cell at a rate of 5 μm per second.

ditions, 100 μM ATP induced a monophasic transient increase in $[Ca^{2+}]_i$ (mean elevation in $[Ca^{2+}]_i$ of 74±10 nM, ± s.e.m., *n*=5), returning to basal levels in approximately 60 seconds. In the presence of 3 mM external Ca^{2+}, application of 100 μM ATP triggered a biphasic Ca^{2+} response, consisting of a sharp rise to a peak value (mean elevation in $[Ca^{2+}]_i$ of 1.2±0.24 μM, ± s.e.m., *n*=5), followed by a rapid decline to an elevated plateau level (Fig. 1B). The elevated Ca^{2+} baseline was maintained for 3-4 minutes before returning to basal levels. This may represent the time taken for the degradation of extracellular ATP to subthreshold concentrations.

In some cell types, extracellular ATP has been shown to promote the formation of pores in the plasma membrane, permeable to ions and small molecular mass molecules (Heppel et al., 1985). However, after treatment with extracellular ATP, PC12 cells remained ≈95% viable (measured using the trypan blue exclusion method) demonstrating that the effects of ATP were not due to cell permeabilisation (results not shown). P_1 purinergic receptors are more effectively activated by adenosine and AMP (Burnstock, 1978). In the present study, adenosine and AMP were ineffective at increasing $[Ca^{2+}]_i$, suggesting that P_2, not P_1, receptors were involved in mediating the ATP-evoked responses (data not shown).

To investigate the changes in $[Ca^{2+}]_i$ in more detail, digital-video imaging was used to study the ATP-induced signal at the single cell level. The responses of four fura-2-loaded individual PC12 cells to an ATP challenge in the presence or absence of external Ca^{2+} are presented in Fig. 1C and D. The traces illustrated are representative of a typical group of single PC12 cells. In the presence of 3 mM external Ca^{2+} (Fig. 1C), 100 μM ATP induced an increase in $[Ca^{2+}]_i$ of between 200 and 600 nM in 112/125 (90%) of cells examined. In contrast, only 36/92 (39%) of single PC12 cells examined responded to 100 μM ATP in the absence of external Ca^{2+} (Fig. 1D). Under these conditions ATP increased $[Ca^{2+}]_i$ to between 10 and 90 nM above the basal level. This smaller rise in $[Ca^{2+}]_i$ was not due to depletion of intracellular Ca^{2+} stores through prolonged incubation in EGTA, since in control experiments, cells released 69% ±13% (*n*=4) of their stored Ca^{2+} after 5 minutes in EGTA; in all the experiments presented in this study, agonists were applied between 30 seconds and 1 minute after EGTA addition.

Table 1. Total [^{3}H]inositol phosphates generated on stimulation with 100 μM ATP, 100 μM UTP or a combination of both agonists in the absence of external Ca^{2+}

External $[Ca^{2+}]$/[EGTA]	Agonist	Total [^{3}H]inositol phosphate accumulated above basal (% of ^{3}H) (*n*=3)
0 mM/1 mM	100 μM ATP	0.094 ± 0.014
0 mM/1 mM	100 μM UTP	0.090 ± 0.030
0 mM/1 mM	100 μM ATP/UTP	0.114 ± 0.035

PC12 populations were preincubated with [^{3}H]inositol for 24 hours prior to stimulation with agonist (see Materials and Methods). Inositol phosphates generated during a 10 minute challenge with ATP, UTP or ATP+UTP were quantified using anion exchange chromatography. Data are mean ± s.e.m. (*n*=3) and represent the increase above basal values. A combination of ATP and UTP does not evoke an additive response, suggesting that they act through the same signalling pathway.

Extracellular ATP induces two different patterns of Ca^{2+} signalling

The spatial characteristics of the ATP-induced Ca^{2+} signals in the presence and absence of external Ca^{2+} were visualised using digital-video imaging of single cells (Fig. 2). Of the 39% of cells that responded to ATP in the absence of external Ca^{2+}, (7/36) 19% of these were observed to respond with a localised increase in $[Ca^{2+}]_i$, which was then propagated through the cell in the form of a wave at a rate of 6.5±1.1 μm/s (*n*=6, Fig. 2A). In the presence of 3 mM external Ca^{2+}, the majority of single PC12 cells filled with Ca^{2+} within 1-2 seconds without any detectable discrete initiation site. However, 10/112 (9%) of cells presented clear examples, where a large subplasmalemmal increase in Ca^{2+} preceded a more global elevation (Fig. 2B).

Extracellular ATP stimulates phosphoinositide hydrolysis

In common with other Ca^{2+}-mobilising agonists, extracellular stimulation by ATP in a variety of cells is associated with G-protein activation of phosphoinositidase C and subsequent hydrolysis of phosphatidylinositol and inositol phosphate accumulation (Charest et al., 1985; Hallam and Pearson, 1986; Sasakawa et al., 1987; Pearce et al., 1989). In order to investigate the possibility that in PC12 cells ATP evoked a similar response, the effect of ATP on the formation of inositol phosphates was determined using anion exchange chromatography. Table 1 presents the total [^{3}H]inositol phosphate accumulated after PC12 populations were incubated with 100 μM ATP or with 100 μM of the pyrimidine nucleotide, UTP. In the absence of external Ca^{2+}, ATP and UTP were almost equipotent in stimulating inositol phosphate production. When cells were challenged with a combined ATP/UTP stimulus the response was not additive, suggesting that both nucleotides were activating identical signalling pathways.

ATP activates two Ca^{2+} signalling pathways

The concentration-response of the rise in $[Ca^{2+}]_i$ induced by extracellular ATP in the presence and absence of external Ca^{2+} is shown in Fig. 3. The concentrations at which ATP induced a half-maximal response, EC_{50}, were 6.5 μM (*n*=3) in the presence of 1 mM excess EGTA, and 32.5 μM (*n*=3) in the presence of 3 mM external Ca^{2+}. This large difference in the EC_{50} values suggested that, in the presence of external Ca^{2+}, ATP may activate an additional pathway, presumably involving Ca^{2+} entry, to increase $[Ca^{2+}]_i$. In order to test whether or not the pathways could be independently activated it was necessary to find a stimulus that would differentially activate one pathway and not the other. We therefore examined the ability of the pyrimidine nucleotide UTP, which was equipotent with ATP at generating inositol phosphates (Table 1), to elicit a Ca^{2+} signal in PC12 cells in the presence and absence of external Ca^{2+}. The EC_{50} for the UTP-induced increase in $[Ca^{2+}]_i$ was observed to be 6.7 μM (*n*=3) in the absence of external Ca^{2+} and interestingly, not significantly different (6 μM, *n*=3) when external Ca^{2+} was present. These data suggest that UTP, in common with ATP, releases intracellularly stored Ca^{2+} but that UTP is unable to activate the additional pathway that is triggered by ATP and revealed in the presence of external Ca^{2+}.

UTP activates a Ca^{2+}-mobilising mechanism in common with ATP

Intact fura-2-loaded PC12 populations were stimulated with 100 μM ATP, 100 μM UTP or a combination of the two agonists. In line with the inositol phosphate response (Table 1), the ATP- and UTP-induced rises in $[Ca^{2+}]_i$ were of similar magnitude (ATP, 74±10 nM (*n*=5); UTP, 62±7 nM (*n*=3), Fig. 4A) and both agents applied simultaneously did not produce an additive response (data not shown). Interestingly, in Ca^{2+}-free conditions, prior treatment with UTP abolished the subsequent Ca^{2+}-mobilising response to ATP (see Fig. 4A) but not the large Ca^{2+} response observed on ATP application in the presence of extracellular Ca^{2+} (Fig. 4B). These results suggest that UTP activates a common receptor with ATP to mobilise intracellular Ca^{2+} but that, in addition, ATP activates an independent receptor, insensitive to UTP, that triggers Ca^{2+} entry.

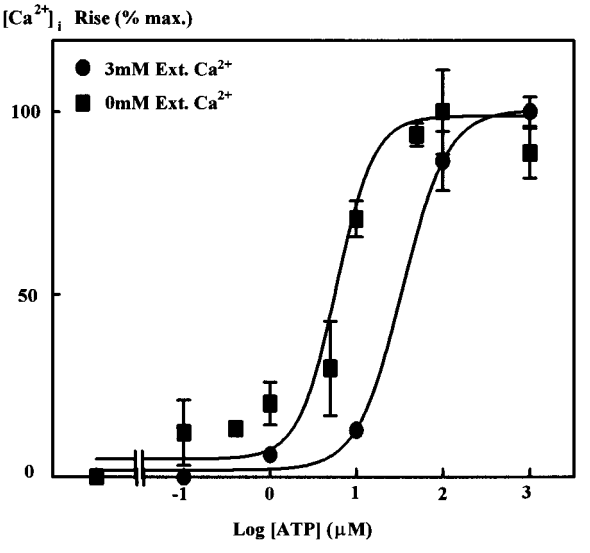

Fig. 3. The concentration-response of the rise in $[Ca^{2+}]_i$ in PC12 populations responding to extracellular ATP in the presence and absence of external Ca^{2+}. An intact fura-2-loaded PC12 population was challenged with ATP in the presence of 3 mM external Ca^{2+} (●), or in the presence of 1 mM excess EGTA (■). ATP was applied between 30 and 60 seconds after the addition of EGTA.

ATP, not UTP, can activate a cation channel permeable to Mn^{2+}

In order to investigate in more detail the relationship between ATP-induced Ca^{2+} mobilisation and Ca^{2+} influx, the Mn^{2+} quench technique (Hallam and Rink, 1985) was used to monitor divalent cation influx into PC12 cells. Mn^{2+} substitute for Ca^{2+} in many Ca^{2+}-permeable pathways but when Mn^{2+} bind to fura-2, instead of an increase in fluorescence intensity, the dye becomes quenched. This quench is best monitored at the isosbestic wavelength of fura-2 (360 nm), where the dye is insensitive to changes in Ca^{2+} concentration. Hence a decrease in fura-2 fluorescence at 360 nm, indicates that Mn^{2+} has entered the cell (there are no Mn^{2+}-containing intracellular compartments) and suggests that a divalent cation-entry pathway has been activated. Intact fura-2-loaded PC12 populations were alternately excited at 340 nm in order to monitor changes in $[Ca^{2+}]_i$, and at 360 nm to monitor Mn^{2+} quenching. An example is shown in Fig. 5A, which illustrates the response to 100 μM ATP in the presence of external Ca^{2+} and external Mn^{2+}. The rise in fluorescence intensity at 340 nm excitation indicated a large increase in $[Ca^{2+}]_i$ on ATP application, and the simultaneous decrease in fluorescence intensity at 360 nm excitation suggested that a divalent-cation entry pathway had been activated. It was suggested that UTP desensitises PC12 populations to ATP-induced mobilisation of intracellular Ca^{2+}, but not ATP-induced influx of external Ca^{2+} (Fig. 4). This is more directly shown in Fig. 5B, in which cells were challenged in nominally Ca^{2+}-free medium containing 200 μM Mn^{2+}.

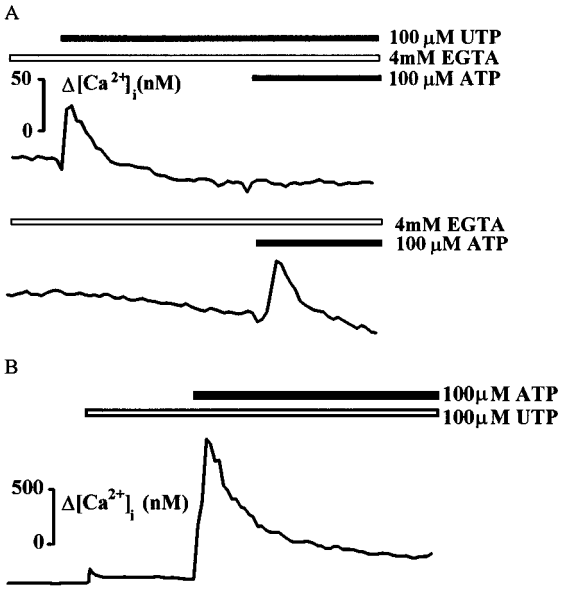

Fig. 4. Population PC12 $[Ca^{2+}]_i$ response to ATP and UTP in the presence and absence of external Ca^{2+}. A fura-2-loaded PC12 cell population was challenged with 100 μM UTP at least 1 minute prior to a subsequent application of 100 μM ATP in the presence of 1 mM excess EGTA (A), or in the presence of 3 mM external Ca^{2+} (B). UTP differentially desensitises the Ca^{2+}-mobilising response to ATP, while leaving the ATP-induced Ca^{2+} influx response uneffected. This provides further evidence to suggest that UTP activates a receptor that is independent of the receptor responsible for activating Ca^{2+} entry.

Addition of 100 μM UTP resulted in a monophasic transient increase in $[Ca^{2+}]_i$ as expected from Fig. 4A. A subsequent challenge with 100 μM ATP did not induce a rise in $[Ca^{2+}]_i$ because, as also shown in Fig. 4A, prior treatment with UTP abolishes this response. However, although ATP did not release intracellularly stored Ca^{2+}, the signal at 360 nm dropped markedly, indicating that a Mn^{2+}-permeable divalent cation entry pathway had nevertheless been activated.

Thus, the use of fura-2 to monitor both intracellular Ca^{2+} (Fig. 4) and intracellular Mn^{2+} (Fig. 5) demonstrated that ATP-induced Ca^{2+} mobilisation and divalent cation entry can be dissociated using the pyrimidine nucleotide UTP. It should be noted also that UTP did not stimulate detectable Mn^{2+} entry (360 nm trace, Fig. 5B), further suggesting that the ATP-activated divalent cation entry is independent of the nucleotide-activated mobilisation of intracellular Ca^{2+}.

ATP-induced Ca^{2+} entry occurs independently of Ca^{2+} mobilisation

In order to confirm that ATP-induced divalent cation entry occurred independently of intracellular Ca^{2+} mobilisation, Mn^{2+} quench experiments were carried out using single cells. The response of a single fura-2-loaded PC12 cell to a challenge

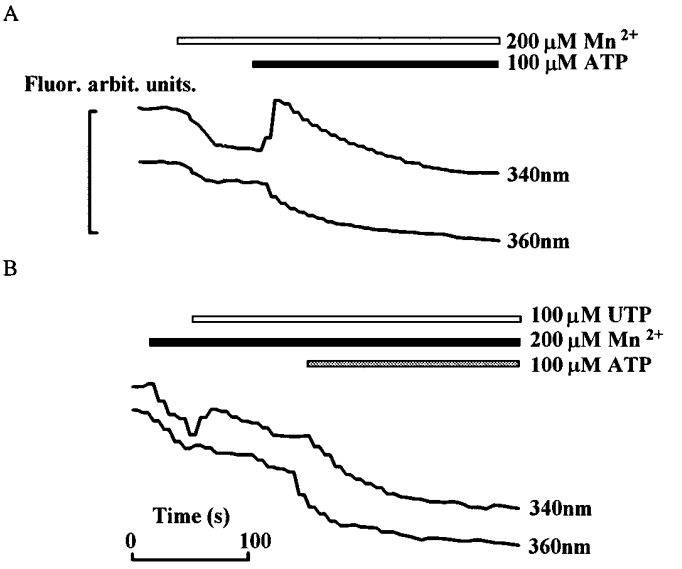

Fig. 5. Mn^{2+} quench studies of PC12 populations in response to ATP and UTP in the presence or absence of external Ca^{2+}. A fura-2-loaded PC12 population was excited at 340 nm to monitor changes in $[Ca^{2+}]_i$, and 360 nm in order to monitor quenching of fura-2 dye by Mn^{2+}. (A) The response to 100 μM ATP in 3 mM external Ca^{2+}. 100 μM ATP triggered an increase in $[Ca^{2+}]_i$ as judged by the increase in signal at 340 nm, and Mn^{2+} influx, as indicated by the decrease in fluorescence at 360 nm, implying that a divalent cation entry pathway had been activated. (B) A population of cells was excited at 340 and 360 nm in nominally Ca^{2+}-free medium; 100 μM UTP triggered the mobilisation of intracellular stores as indicated by the increase in fluorescence at 340 nm, but no Mn^{2+} influx. A subsequent challenge with ATP did not result in a mobilisation response but nevertheless triggered a large Mn^{2+} influx. Therefore it appeared that (i) UTP desensitised ATP-induced Ca^{2+} mobilisation while leaving ATP-induced influx uneffected; and (ii) UTP did not trigger a Mn^{2+}-permeable influx pathway itself.

with 100 μM ATP in the presence of external Ca^{2+} and 200 μM Mn^{2+} is shown in Fig. 6A. This response confirmed the result obtained from cell populations presented in Fig. 5A; ATP induced a rise in $[Ca^{2+}]_i$ and activated a large influx of Mn^{2+}. As discussed earlier (Fig. 1C and D), only 40% of single PC12 cells responded with a rise in $[Ca^{2+}]_i$, to ATP in the absence of extracellular Ca^{2+}. An example of one of the 60% of cells that did not respond to ATP under Ca^{2+}-free conditions with a Ca^{2+}-mobilisation response is shown in Fig. 6B. Interestingly, on application of ATP, the fluorescence at 360 nm was rapidly quenched, indicating that although Ca^{2+} mobilisation was not activated, stimulation of the Mn^{2+}-permeable entry pathway still occurred. Hence, ATP-induced activation of divalent cation influx does not require the depletion of intracellular stores or a rise in intracellular Ca^{2+}. In addition, the large ATP-activated divalent cation entry component does not appear to be stimulated as a result of phosphoinositide hydrolysis because UTP stimulated inositol phosphate generation to the same extent as ATP (Table 1), but did not activate the Mn^{2+}-permeable Ca^{2+} entry pathway (Fig. 5B).

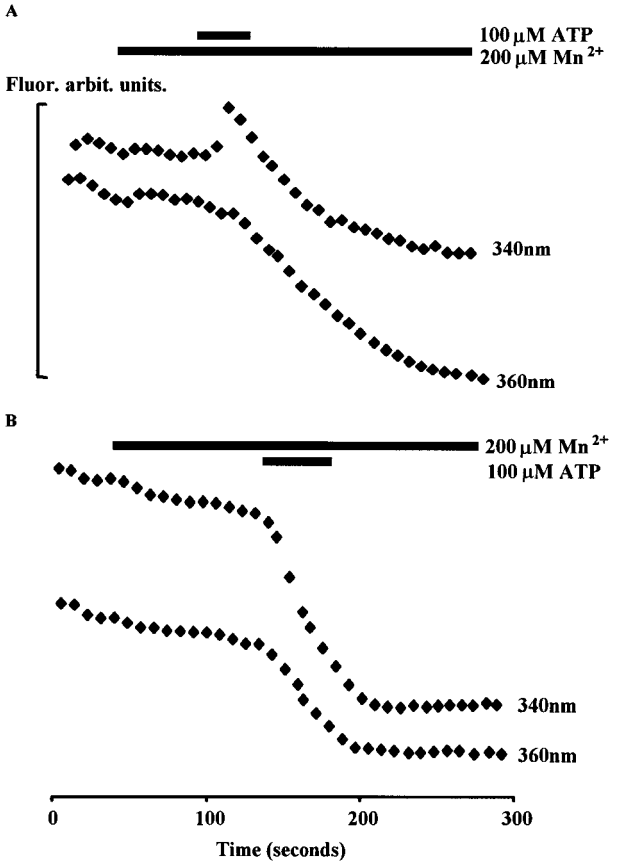

Fig. 6. Single cell Mn^{2+} quench stimulated on addition of extracellular ATP in the presence and absence of external Ca^{2+}. Digital-video imaging of fura-2-loaded single PC12 cells was combined with the technique of Mn^{2+} quench. The response of a single cell to 100 μM ATP in the presence of 3 mM external Ca^{2+} is presented in (A). The increase in fluorescence at 340 nm indicated a rise in $[Ca^{2+}]_i$, whilst the decrease in intensity at 360 nm indicated stimulation of Mn^{2+} influx. The experiment was repeated in nominally Ca^{2+}-free medium using a cell that did not respond to ATP with a Ca^{2+}-mobilisation response (B). ATP still induced Mn^{2+} entry, suggesting that the mechanism triggering divalent cation influx is independent of Ca^{2+} store mobilisation.

ATP-induced Ca^{2+} entry is independent of L-type voltage-operated channels

The predominant voltage-operated Ca^{2+} channel (VOC) present on undifferentiated PC12 cells is the L-type channel (Plummer et al., 1989). L-type VOCs are sensitive to dihydropyridine antagonists, so in order to investigate the contribution of VOCs to ATP-activated Ca^{2+} entry, intact fura-2-loaded cell populations were preincubated for 10 minutes with the dihydropyridine antagonist, nifedipine (10 μM), prior to activation (Fig. 7A and B). As expected, nifedipine pretreatment markedly attenuated the Ca^{2+} rise induced by depolarising concentrations of 55 mM K^+ (77±18%, *n*=3, Fig. 7B). However, under the same conditions the ATP-induced response was reduced by only 24±8% (*n*=3), indicating that although L-type VOCs may contribute to the signal, the majority of the ATP-induced Ca^{2+} entry occurs *independently* of L-type channel activation.

ATP-induced Ca^{2+} entry, but not mobilisation of intracellular Ca^{2+}, triggers secretion

An elevation in $[Ca^{2+}]_i$ triggers catecholamine secretion from PC12 cells (Appel and Barefoot, 1989). In order to measure secretion, PC12 cells were preloaded with [^{3}H]dopamine before nucleotide stimulation. The concentration-response of

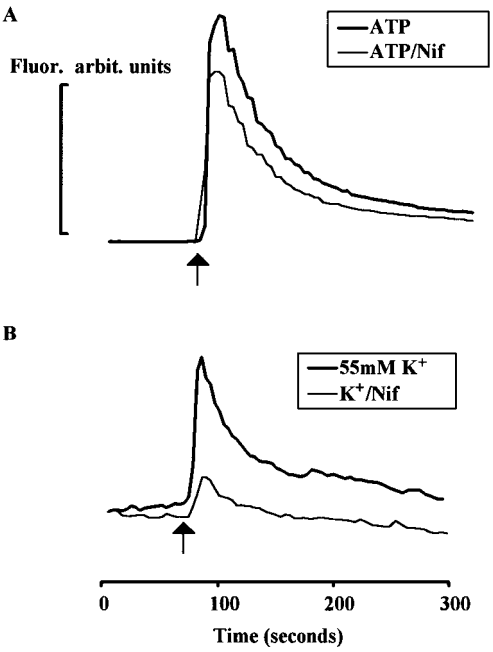

Fig. 7. The effect of the dihydropyridine antagonist, nifedipine (Nif) on the $[Ca^{2+}]_i$ rise induced by extracellular ATP and depolarising concentrations of K^+. fura-2-loaded PC12 populations were pretreated with 10 μM nifedipine for 10 minutes prior to challenging with 100 μM ATP or 55 mM K^+. The response to ATP was only reduced by 24±8% (*n*=3) (A), whereas the response to 55 mM K^+ was reduced by 77±18% (*n*=3) (B). ATP-induced Ca^{2+} influx is largely independent of L-type voltage-operated channels.

ATP-induced secretion in the presence and absence of external Ca^{2+} is shown in Fig. 8A; 100 μM ATP (a maximal concentration) evoked secretion of 15.8±4.4% (n=7) [^{3}H]dopamine above basal levels, but only in the presence of external Ca^{2+}. In the absence of external Ca^{2+}, there was no detectable secretory response at any concentration of ATP tested (Fig. 8A). Further evidence that release of intracellularly stored Ca^{2+} is unable to promote secretion is shown in Fig. 8B. UTP, which generates inositol phosphates (Table 1), mobilises intracellular Ca^{2+} (Fig. 4A) but does not promote Mn^{2+} entry (Fig. 5B), was unable to evoke secretion at any concentration tested in the presence or absence of external Ca^{2+}. These data indicate that the wave of Ca^{2+} evoked on activation of the UTP-sensitive ATP receptor (Fig. 2A) is an insufficient signal to trigger transmitter release. Instead, ATP-induced Ca^{2+} entry, which elevates $[Ca^{2+}]_i$ directly underneath the plasma membrane (Fig. 2B), appears to be the important signal for secretion.

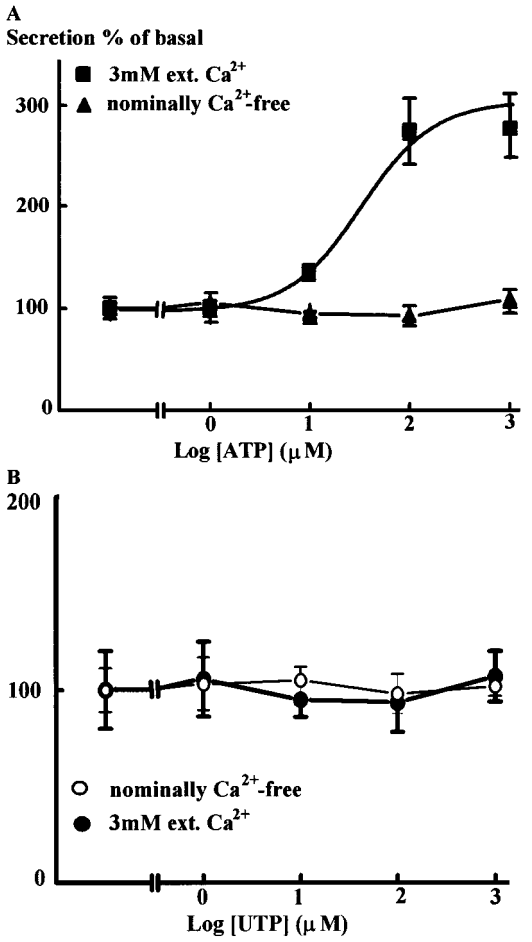

Fig. 8. Extracellular ATP triggers [^{3}H]dopamine release only in the presence of external Ca^{2+}. UTP is unable to evoke transmitter release under any conditions. PC12 populations were preloaded with [^{3}H]dopamine as described in Materials and Methods. Secretion presented as a percentage of the basal value (100%). ATP-induced secretion in the presence of 3 mM external Ca^{2+} (■) is compared with secretion in the absence of external Ca^{2+} (▲) (A). ATP triggered secretion only in the presence of external Ca^{2+}, with an EC_{50} of 23 μM (n=7). Challenging PC12 populations with extracellular UTP did not evoke a secretory response at any concentration (B). These results indicate that intracellular Ca^{2+} mobilisation triggered by activation of the nucleotide receptor is insufficient to evoke secretion. Instead, activation of the independently triggered Ca^{2+} influx pathway is absolutely required for the onset of transmitter release.

ATP-induced secretion can be localised to the same region as divalent cation entry

In view of their important role in the secretory response, we visualised the distribution of divalent cation entry sites at the single cell level using digital-video imaging together with Mn^{2+} quench. Monitoring Mn^{2+} quenching of the ratio image (340 nm/380 nm) overcame any inconsistencies in fura-2 loading within the cell, while also allowing a simultaneous indication of any increases in $[Ca^{2+}]_i$ (see Materials and Methods; Cheek et al., 1993). Immediately after the addition of 100 μM ATP, fura-2 fluorescence began to quench progressively around the whole subplasmalemmal region of the cell, presenting the illusion that the cell was decreasing in size (Fig. 9A). This effect was due to Mn^{2+} entering the cell and quenching fura-2 initially in the cortical region, and it indicated that extracellular ATP activated divalent cation entry over the entire cell surface.

Exocytotic sites were localised by following the distribution of the secretory granule membrane protein, dopamine-β-hydroxylase. PC12 cells adhered to glass coverslips were treated with 100 μM ATP in the presence of 3 mM external Ca^{2+}. After incubation with an antibody to dopamine-β-hydroxylase, and fluorescent labelling with streptavidin-labelled Texas Red, the pattern of secretion was visualised using fluorescence microscopy. The results show that anti-dopamine-β-hydroxylase fluorescence is localised around the entire cell periphery in virtually all cells (Fig. 9B). This figure therefore indicates how both divalent cation influx *and* exocytosis are co-localised to the entire cell periphery, providing a further indication as to the close inter-relationship between divalent cation influx and the triggering of secretion.

DISCUSSION

PC12 cells represent an ideal model system in which to study, not only the role played by extracellular and intracellular Ca^{2+} in mediating exocytotic secretion, but also the regulatory effects that various neuromodulatory substances have on the secretory response. One such neuromodulator is extracellular ATP, which is co-released from PC12 cells (Roskoski and Roskoski, 1989), adrenal chromaffin cells (Rojas et al., 1985; White et al., 1987) and nerve terminals in both the peripheral and central nervous systems (Potter and White, 1980; Silinsky and Ginsborg, 1983; Richardson and Brown, 1987). In many cell types, extracellular ATP elicits a transient elevation in $[Ca^{2+}]_i$, and there is general agreement that these effects are mediated by nucleotide-specific receptors (Burnstock, 1978; Kennedy, 1990).

Extracellular ATP generates complex patterns of $[Ca^{2+}]_i$

Addition of extracellular ATP to populations of PC12 cells evoked distinct Ca^{2+} signals, depending on the extracellular environment. In the absence of external Ca^{2+}, ATP triggered a

small transient elevation in $[Ca^{2+}]_i$, which returned to basal levels within 1 minute. However, in the presence of external Ca^{2+}, ATP evoked a much larger increase in $[Ca^{2+}]_i$, which remained at an elevated plateau level. These data suggest that ATP stimulates both the mobilisation of intracellular Ca^{2+} and the influx of external Ca^{2+} in these cells, and are consistent with findings obtained using fura-2-loaded PC12 cells (Fasolato et al., 1990) and cells activated in the presence of $^{45}Ca^{2+}$ (Sela et al., 1991). Video-imaging of single fura-2-loaded cells, however, revealed that the population measurements of $[Ca^{2+}]_i$ masked considerable heterogeneity at the single cell level; in the presence of external Ca^{2+}, 90% of cells responded with an elevation in $[Ca^{2+}]_i$, whereas only 39% responded in the absence of external Ca^{2+}. Similar single cell heterogeneity has been observed in other PC12 clones (Grohovaz et al., 1991) and in their non-tumorigenic counterpart, adrenal chromaffin cells (O'Sullivan et al., 1989; Kim and Westhead, 1989). In the present study, the subcellular spatial organisation of the Ca^{2+} signal was also found to vary, depending upon the presence or absence of external Ca^{2+}. In the absence of extracellular Ca^{2+}, a wave of Ca^{2+} that propagated from a discrete polarised initiation site was detected in 19% of cells (Fig. 2A), whereas in the presence of external Ca^{2+} the rise in $[Ca^{2+}]_i$ was often observed to initiate exclusively in the subplasmalemmal region (Fig. 2B) prior to a more global increase in $[Ca^{2+}]_i$. The reasons why these complex spatial patterns of $[Ca^{2+}]_i$ were only observed in a sub-population of cells are not clear. One contributory factor is likely to be the resolution of the imaging system, which is limited to an aquisition rate of 5 ratio images/second. For example, it is possible that all the cells responding to ATP in the absence of external Ca^{2+} display a Ca^{2+} wave, but that the initiation site

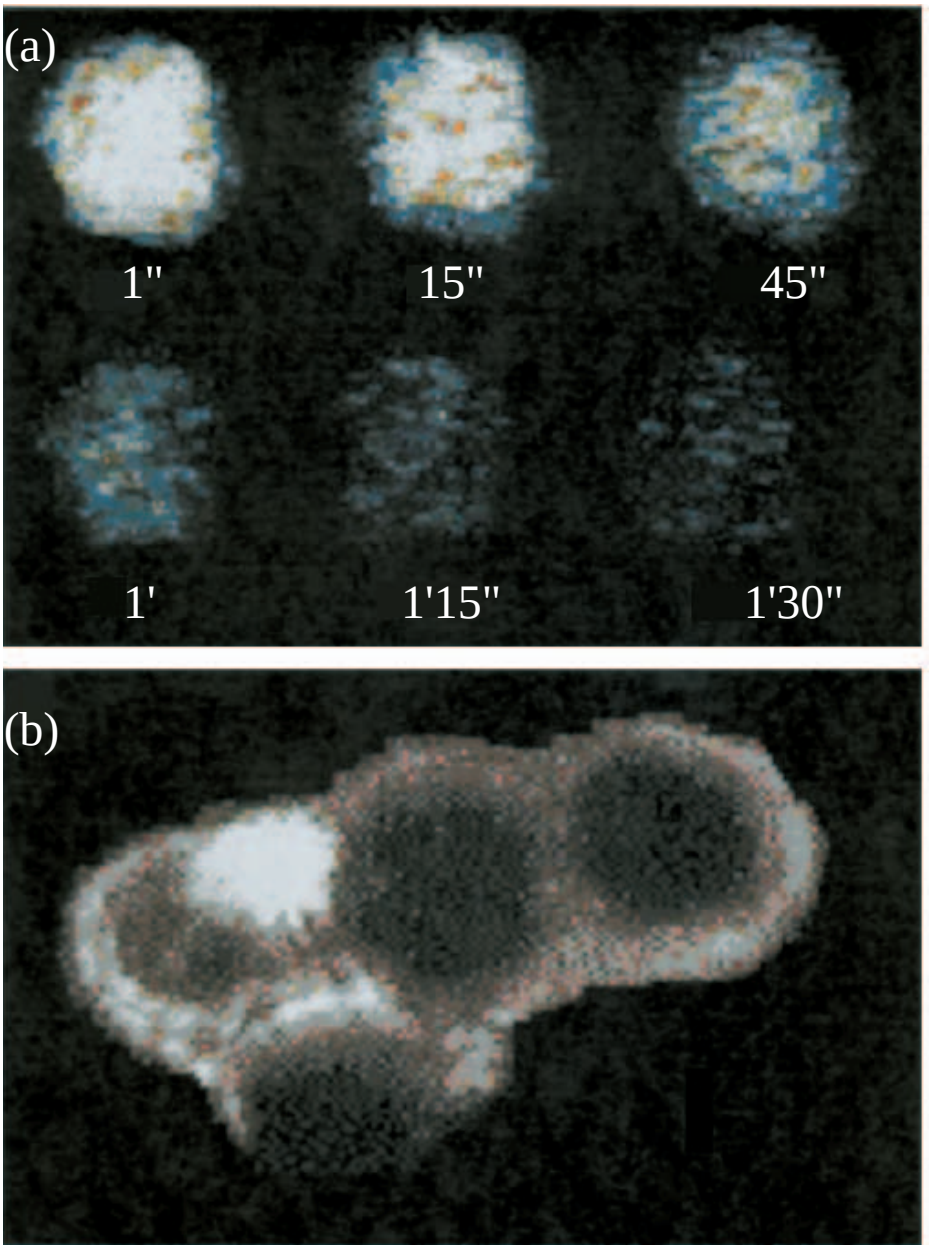

Fig. 9. Single cell visualisation of the subcellular site of divalent cation entry indicated by Mn^{2+} influx, and of exocytosis by mapping the distribution of antibodies to dopamine-β-hydroxylase. fura-2-loaded single PC12 cells were challenged with 100 μM ATP after the addition of 200 μM Mn^{2+} to external medium containing 3mM Ca^{2+} (a). The montage presents a series of 6 single frames taken from 1 second to 1 minute 30 seconds after stimulation. After application of ATP, the fura-2 signal was progressively quenched around the entire cell periphery, indicating that Mn^{2+} entry had been stimulated. The distribution of antibody to the secretory granule membrane protein dopamine-β-hydroxylase is illustrated in (b), and highlights the localisation of secretion. Notice how influx and transmitter release are co-localised to the same plasmalemmal region of the cell.

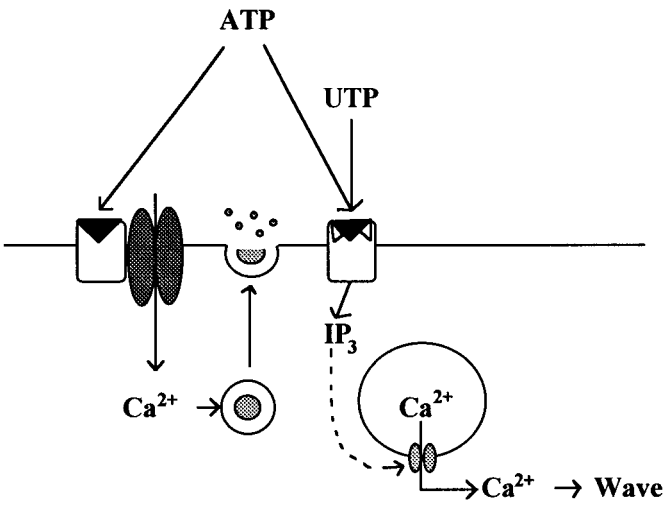

Fig. 10. Scheme showing receptor-mediated intracellular events that could account for ATP-induced Ca^{2+} signals and secretory responses in PC12 cells. Activation of a nucleotide receptor by UTP and ATP releases intracellularly stored Ca^{2+}, often in the form of a wave, that is unable to promote secretion. Activation of Ca^{2+} entry through an independently regulated divalent cation channel stimulates secretion because $[Ca^{2+}]_i$ is sufficiently elevated in the subplasmalemmal region of the cell.

is only in the plane of focus in ~20% of cells at any one time. It is unlikely, however, that such an explanation could entirely account for the heterogeneity we observed, and an additional possibility that we examined was that ATP could activate more than one Ca^{2+}-signalling pathway in these cells, perhaps by activating more than one class of cell surface receptor.

Extracellular ATP generates inositol phosphates

Purinergic receptors of the P_{2y} subtype that are coupled to the hydrolysis of phosphatidylinositol are widely distributed in many cell types (Kennedy and Burnstock, 1985; Haggblaad and Heilbron, 1987; Dubyak et al., 1988; Cooper et al., 1989; Forsberg et al., 1991), but their association with PC12 cells is controversial, possibly because different PC12 cell clones appear to have different biochemical characteristics (Grohovaz et al., 1991). For example, ATP acting through P_{2y}-like receptors has been reported to be able to mobilise intracellularly stored Ca^{2+} in some PC12 clones (Fasolato et al., 1990; Clementi et al., 1992) but not others (Raha et al., 1993). In the PC12 clone available in our laboratory, extracellular ATP was able to generate inositol phosphates (Table 1) and release intracellularly stored Ca^{2+} (Figs 1 and 2), which would be consistent with the presence of a P_2 receptor. However, in our study, the same receptor was also activated by pyrimidine nucleotides, because UTP generated an ATP-like inositol phosphate signal (Table 1) and a rise in $[Ca^{2+}]_i$ (Fig. 4) that were not additive with the ATP-induced responses. UTP-sensitive receptors have been observed in a number of cell types such as fibroblasts (Fine et al., 1989) and HL60 cells (Stutchfield and Cockcroft, 1990) as well as PC12 cells (Raha et al., 1993), and this has led to the suggestion that ATP and UTP may act through a common 'nucleotide' receptor that is distinct from the conventional P_{2y} receptor (Pfeilschifter, 1990; Brown et al., 1991; Lin et al., 1993). One possibility is that the receptor is a form of the recently identified P_{2u} receptor that has been shown to mobilise intracelluar Ca^{2+} in response to activation by ATP or UTP (Lustig et al., 1993).

Extracellular ATP promotes Ca^{2+} influx

The influx of Ca^{2+} stimulated by ATP (Fig. 2B) was further revealed by monitoring the quench of fura-2 induced by the influx of the Ca^{2+} surrogate, Mn^{2+} (Figs 5 and 6) and also by visualising this quench (Fig. 9). Agonist-stimulated hydrolysis of phosphatidylinositol is accompanied by Ca^{2+} influx in many cell types (for a recent review see Penner et al., 1993), but there are several reasons for supposing that the ATP-induced entry of divalent cations observed in this study does not require mobilisation of intracellular Ca^{2+} and is mediated by an ATP-specific receptor that is independent of the UTP-sensitive nucleotide receptor. Firstly, both UTP and ATP generated similar amounts of inositol phosphates (Table 1), but only ATP promoted Mn^{2+} entry (Fig. 5). Secondly, UTP pretreatment desensitised ATP-induced intracellular Ca^{2+} mobilisation, but not ATP-induced influx of Ca^{2+} (Fig. 4) or Mn^{2+} (Fig. 5). Thirdly, single cell experiments revealed a subpopulation of cells in which ATP stimulated divalent cation entry (Fig. 6) without promoting mobilisation of intracellular Ca^{2+}. Lastly, short-term pretreatment (10 minutes) with the phorbol ester PMA, inhibited inositol phosphate production and intracellular Ca^{2+} mobilisation induced by ATP and UTP, but did not effect ATP-induced Ca^{2+} entry (data not shown). All these data suggest that ATP activates a divalent cation entry pathway in PC12 cells, which is consistent with $^{45}Ca^{2+}$ uptake studies (Sela et al., 1991) and studies on ATP-activated inward currents in PC12 cells (Nakazawa et al., 1990; Neuhaus et al., 1991; Reber et al., 1992; Nakazawa and Hess, 1993), and that in addition ATP triggers these effects independently of its ability to mobilise intracellular Ca^{2+}. Just how ATP promotes divalent cation entry is unknown. PC12 cells express L-type voltage-operated Ca^{2+} channels (Plummer et al., 1989), but concentrations of the dihydropyridine antagonist nifedipine that significantly attenuated a depolarising K^+-induced Ca^{2+} signal were relatively ineffective at inhibiting an ATP-induced Ca^{2+} signal (Fig. 7), arguing against a significant role for L-type voltage-operated channels. Previous studies have reported either very little (Raha et al., 1993) or a significant (~40%, Fasolato et al., 1990) contribution of voltage-operated Ca^{2+} entry to the ATP-induced elevation in $[Ca^{2+}]_i$ in PC12 cells. However, it is clear that the majority of the ATP-stimulated Ca^{2+} entry into PC12 cells is through a non-voltage-dependent pathway. One possibility is that ATP activates a receptor-operated non-selective cation channel, as reported in smooth muscle cells (Benham and Tsien, 1987) and sensory neurones (Friel and Bean, 1988; Bean, 1990; Bean et al., 1990;), because a channel with similar properties has been identified using outside-out patches of PC12 cells (Neuhaus et al., 1991).

Ca^{2+} influx, but not the release of intracellularly stored Ca^{2+}, triggers secretion

Our results suggest that ATP is capable of activating two independent Ca^{2+} signalling pathways in PC12 cells, and that distinctive patterns of Ca^{2+} are delivered into the cell by these two pathways; activation of the UTP-sensitive nucleotide receptor releases intracellularly stored Ca^{2+} and often triggers a Ca^{2+}

wave, whereas activation of the putative ATP-sensitive receptor-operated channel triggers Ca^{2+} entry and an initial subplasmalemmal localisation of $[Ca^{2+}]_i$. In order to investigate whether these distinctive Ca^{2+} signals were distinguishable at a functional level, the exocytotic secretion of [^{3}H]dopamine was measured after stimulation of the cells with ATP and UTP in the presence and absence of external Ca^{2+}. Secretion induced by ATP was absolutely dependent upon the presence of external Ca^{2+}, and UTP was ineffective at triggering secretion, irrespective of whether or not external Ca^{2+} was present (Fig. 8). These data suggest that only Ca^{2+} entry, not the release of intracellular Ca^{2+}, can trigger secretion. Others have also observed that ATP-induced secretion from PC12 cells is dependent on external Ca^{2+} (Inoue et al., 1989; Sela et al., 1991). Our data provide a possible explanation for these results; activating Ca^{2+} entry delivers Ca^{2+} directly to the subplasmalemmal region of the cell, which is then ideally localised to activate the exocytotic sites. Indeed, the finding that the ATP-induced subcellular sites of divalent cation entry co-localised with the sites of exocytosis (Fig. 9) confirmed that a close inter-relationship exists between divalent cation entry and secretion in these cells. Ca^{2+} entry appears to play a key role in promoting secretion from other tissues that release fast neurotransmitters such as adrenal chromaffin cells (Augustine and Neher, 1992a; Cheek et al., 1993), pituitary cells (Thomas et al., 1993) and cells of the squid giant synapse (Llinas et al., 1992), and this has lead to the proposal that a very high (10-100 μM) elevation of Ca^{2+} in a localised region of the cell just beneath the plasma membrane is the prime signal for activating the fusion complex (Augustine and Neher, 1992b; Cheek and Barry, 1993). In contrast, delivering Ca^{2+} into the cell in the form of a Ca^{2+} wave appears not to be able to activate fusion, presumably because $[Ca^{2+}]_i$ is not elevated to sufficient magnitude at the subplasmalemmal exocytotic sites (Cheek and Barry, 1993).

Conclusions

We have shown that one stimulus, extracellular ATP, can evoke Ca^{2+} signals with different temporal and spatial patterns in PC12 cells by activating independent Ca^{2+} signalling pathways and, importantly, that these Ca^{2+} signals can be functionally distinct.

Activation of the putative nucleotide receptor, results in phosphoinositide hydrolysis and intracellular Ca^{2+} mobilisation, which has been visualised as a Ca^{2+} wave in some cells. Extracellular ATP-induced Ca^{2+} waves have also been observed in porcine aortic smooth muscle cells (Mahoney et al., 1993). Currently the most favoured mechanism to explain intracellular Ca^{2+} wave propagation involves a process of Ca^{2+}-induced Ca^{2+} release (CICR), which may be mediated via the InsP_3 receptor or ryanodine receptor (Berridge, 1993). In the PC12 clone under current investigation, experiments suggest that both receptor types exist, and therefore ATP-induced Ca^{2+} waves may propagate by a process of CICR through either receptor (V. A. Barry and T. R. Cheek, unpublished observations). The magnitude and spatial pattern of the Ca^{2+} response induced by activation of the nucleotide receptor is not sufficient to trigger catecholamine secretion, but could regulate other important Ca^{2+}-dependent processes such as synthesis of biosynthetic enzymes and peptide precursors.

Conversely, ATP-activation of the divalent-cation influx pathway, *is* capable of eliciting secretion, presumably by elevating subplasmalemmal Ca^{2+} concentrations to levels sufficient to trigger the secretory fusion apparatus. This has important implications for a role for extracellular ATP in modulating exocytosis triggered by other secretory stimuli, especially when noted that the level of intragranular ATP is high enough to allow external ATP to transiently reach high concentrations after exocytosis.

It will be important to determine whether the UTP-sensitive nucleotide receptors and the putative receptor-operated channels that mediate the Ca^{2+} signals co-exist on the same cells or are uniquely present on biochemically distinct sub-populations of cells. Single cell imaging of fura-2 revealed distinct sub-populations of cells (Grohovaz et al., 1991). In the present study we observed that 90% of single cells responded to ATP with a rise in $[Ca^{2+}]_i$ in the presence of external Ca^{2+}, whereas 39% of cells responded in the absence of external Ca^{2+}, suggesting that there is some overlap in the two responses (Fig. 10). Further work will be required to investigate this, and its functional consequences in greater detail.

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