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Regulation of brain capillary endothelial cells by P2Y receptors coupled to Ca^{2+}, phospholipase C and mitogen-activated protein kinase

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1 The blood-brain barrier is formed by capillary endothelial cells and is regulated by cell-surface receptors, such as the G protein-coupled P2Y receptors for nucleotides. Here we investigated some of the characteristics of control of brain endothelial cells by these receptors, characterizing the phospholipase C and Ca^{2+} response and investigating the possible involvement of mitogen-activated protein kinases (MAPK).

2 Using an unpassaged primary culture of rat brain capillary endothelial cells we showed that ATP, UTP and 2-methylthio ATP (2MeSATP) give similar and substantial increases in cytosolic Ca^{2+}, with a rapid rise to peak followed by a slower decline towards basal or to a sustained plateau. Removal of extracellular Ca^{2+} had little effect on the peak Ca^{2+}-response, but resulted in a more rapid decline to basal. There was no response to α,β-MethylATP (α,βMeATP) in these unpassaged cells, but a response to this P2X agonist was seen after a single passage.

3 ATP (log EC_{50} -5.1 ± 0.2) also caused an increase in the total [^{3}H]-inositol (poly)phosphates ([^{3}H]-$InsP_x$) in the presence of lithium with a rank order of agonist potency of ATP = UTP = UDP > ADP, with 2MeSATP and α,βMeATP giving no detectable response.

4 Stimulating the cells with ATP or UTP gave a rapid rise in the level of inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$), with a peak at 10 s followed by a decline to a sustained plateau phase. 2MeSATP gave no detectable increase in the level of $Ins(1,4,5)P_3$.

5 None of the nucleotides tested affected basal cyclic AMP, while ATP and ATPγS, but not 2MeSATP, stimulated cyclic AMP levels in the presence of 5 μM forskolin.

6 Both UTP and ATP stimulated tyrosine phosphorylation of p42 and p44 mitogen-activated protein kinase (MAPK), while 2MeSATP gave a smaller increase in this index of MAPK activation. By use of a peptide kinase assay, UTP gave a substantial increase in MAPK activity with a concentration-dependency consistent with activation at $P2Y_2$ receptors. 2MeSATP gave a much smaller response with a lower potency than UTP.

7 These results are consistent with brain endothelial regulation by $P2Y_2$ receptors coupled to phospholipase C, Ca^{2+} and MAPK; and by $P2Y_1$-like (2MeSATP-sensitive) receptors which are linked to Ca^{2+} mobilization by a mechanism apparently independent of agonist stimulated $Ins(1,4,5)P_3$ levels. A further response to ATP, acting at an undefined receptor, caused an increase in cyclic AMP levels in the presence of forskolin. The differential MAPK coupling of these receptors suggests that they exert fundamentally distinct influences over brain endothelial function.

Keywords: P2Y receptors; P2 receptors; nucleotide receptors; $Ins(1,4,5)P_3$; Ca^{2+} mobilization; endothelial cells; brain endothelial cells; blood-brain barrier; inositol (poly)phosphates; MAPK

Introduction

Following the pioneering work of Burnstock and colleagues it is now accepted that extracellular nucleotides (principally the purines adenosine 5′-triphosphate (ATP) and ADP, and the pyrimidines uridine 5′-triphosphate (UTP) and UDP) exert a widespread influence on bodily function by acting on P2 receptors (Boarder *et al*., 1995; Burnstock, 1996). These P2 receptors are divided into the intrinsic ion channel P2X receptors and the heterotrimeric G protein-coupled P2Y receptors. Cloning and pharmacological studies have defined several members of these subfamilies (e.g. $P2Y_{1\text{-}7}$), while other pharmacological work indicates that there are further, as yet uncloned and unclassified, P2Y receptors.

There have recently been a variety of studies on brain endothelial cells (mainly on passaged cells or immortalized cell lines) investigating the role of various receptors. There are a number of reasons for considering regulation of brain endothelial cells by P2 receptors to be important. The special characteristics of these cells are maintained in part by their complex environment; a role for astrocytes in this process has been established, and astrocytes release ATP. Revest *et al*. (1991) have shown that ATP increases cytosolic calcium concentration ($[Ca^{2+}]_i$) in rat cultured brain capillary endothelial cells (RBEC). Subsequent studies have shown that some combination of P2X and P2Y (including $P2Y_2$, formerly known as P2U) receptors could be found on the cultured cells (e.g. Vigne *et al*., 1994; Nobles *et al*., 1995). In a clonal cell line derived from RBECs it has been shown (Webb *et al*., 1996) that an 'atypical $P2Y_1$' receptor (apparently identical in sequence to the previously cloned rat $P2Y_1$) is coupled to the inhibition of adenylyl cyclase as well as an increase in $[Ca^{2+}]_i$. Furthermore, in these cells $P2Y_1$-like receptors produce an unusual response to stimulation with ADP and 2-methylthioATP (2MeSATP), a Ca^{2+} elevation which does not coincide with a detectable increase in total [^{3}H]-inositol (poly)phosphates ([^{3}H]-$InsP_x$) in [^{3}H]-inositol labelled cells

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(Frelin *et al*., 1993; Vigne *et al*., 1994), implying that there is an Ins(1,4,5)P_3-independent Ca^{2+} mobilization. However, levels of Ins(1,4,5)P_3 were not measured in these earlier studies.

In recent work we have established the importance of tyrosine kinase/mitogen-activated protein kinase (MAPK) cascades in the regulation of major vessel endothelial cell prostacyclin production by P2Y receptors (Bowden *et al*., 1995; Patel *et al*., 1996b). It is known that MAPK cascades, with focal adhesion kinases, are important in cell adhesion in general, and there is well established evidence indicating a role for tyrosine phosphorylation of proteins involved in maintenance of the blood-brain barrier (Staddon & Rubin, 1996), and more generally in the regulation of capillary permeability (Staddon *et al*., 1995). Here we have further characterized the P2Y responses of brain microvascular endothelial cells, with respect to phosphoinositide hydrolysis, cytosolic Ca^{2+} and adenosine 3′:5′-cyclic monophosphate (cyclic AMP). In addition we have investigated the stimulation of p42 and p44 MAPK tyrosine phosphorylation by Western blot and a peptide kinase assay (Wilkie *et al*., 1996) as an index of MAPK activation. The results show that co-existing P2Y receptors may differentially regulate phosphoinositide hydrolysis, cyclic AMP and MAPK in brain endothelial cells, implying that they will exert fundamentally different influences in the regulation of the blood-brain barrier.

Methods

Cell culture

RBECs were prepared from adult Wistar rats by an adaptation of the method described by Rubin (1991) for porcine brain. Cerebral cortices from freshly killed rats were cleaned of meninges, chopped and homogenized in ice-cold buffer (HEPES-buffered M199, 10% foetal calf serum, 1% penicillin-streptomycin) with a glass-glass Wheaton homogenizer, with 89–127 μm clearance followed by 25–76 μm clearance. The resulting slurry was sequentially filtered through 150 μm and 60 μm nylon mesh, and the filtrate collecting on the 60 μm mesh digested for 1 h in enzyme solution consisting of homogenizing buffer with 1 mg ml^{-1} collagenase/dispase (Boehringer-Mannheim, East Sussex, U.K.), and 20 u ml^{-1} DNase (Sigma, Poole, Dorset, U.K.). Capillaries were then plated into 96 well plates for the [^{3}H]-InsP_x or cyclic AMP assays, into 24 well plates for the mass Ins(1,4,5)P_3 assay, 3.5 cm dishes for the Western blots and on glass coverslips for the Ca^{2+} measurements. The cells formed a confluent monolayer with diffuse immunoreactivity for Factor VIII. Cells were used at confluence except for the Fura 2 imaging experiments, when individual plaques of preconfluent cells were studied.

Inositol polyphosphates

For [^{3}H]-InsP_x measurements cells were labelled with *myo*-[^{3}H]-inositol (37 kBq ml^{-1}; Amersham, Bucks, U.K.) in 200 μl of M199 $\pm$ 25 mM HEPES, 1% FCS, 1% penicillin-streptomycin, for 24–48 h before stimulation. Cells were stimulated in the presence of 10 mM LiCl for 16 min, followed by extraction of the [^{3}H]-inositol phosphates into 0.5 mM trichloroacetic acid and separation of [^{3}H]-InsP_x on Dowex-1 Cl^- columns. For hexokinase treatment to remove triphosphonucleotides (Harden *et al*., 1977), UDP stock (5 mM) was preincubated for 1 h with 50 u ml^{-1} hexokinase (Sigma) and 110 mM glucose. Hexokinase was present at 10 u ml^{-1} during the stimulation of the cells.

Mass Ins(1,4,5)P_3 was measured in trichloroacetic acid extracts by the method of Challiss *et al*. (1988).

Cytosolic Ca^{2+}

Cytosolic Ca^{2+} ($[Ca^{2+}]_i$) was monitored in Fura 2 imaging experiments. Preconfluent cells were loaded with 2 μM Fura 2 AM (Calbiochem, Nottingham, U.K.) for 30 min at room temperature with 1% bovine serum albumin, and fluorescent images at 340 and 380 nm excitation wavelengths were captured by an ImproVision microspectrofluorimetry system. Cells were continuously perfused and drugs applied with a multibarrelled perfusion system. Ratio data were collected for each individual cell in the field (normally 15–35 cells) and in some cases is shown for individual cells while in other cases data are pooled across cells. Data are presented as the 340/380 ratio.

Western blots

Western blots were performed on extracts from cells maintained in 1% serum for the previous 24 h, stimulated for 5 min and extracted into 20 mM Tris (pH 7.4), 2 mM EDTA, 10 μg ml^{-1} leupeptin, 20 μM E-64, 2 μg ml^{-1} aprotinin, 1 μM pepstatin A, 50 mM sodium fluoride, 2.5 mM sodium orthovanadate, 62.5 mM β-glycerophosphate, 1 mM phenylmethylsulphonylfluoride and 0.1% Triton X-100. After separation on 10% SDS-polyacrylamide gels proteins were blotted onto nitrocellulose, probed with 1:500 dilution of antiserum specific for p42/p44 MAPK phosphorylated at tyrosine-204 and visualized by the enhanced chemilumin-escence (ECL) procedure of Amersham. The use of this procedure for Western blotting of the tyrosine phosphorylated forms of the MAPK enzymes has been described previously (Wilkie *et al*., 1996; Patel *et al*., 1996b).

Peptide kinase assay

The activity of p42/p44 MAPK was measured with [^{32}P]-ATP and a nonapeptide designed to be a preferential substrate for MAPK, as described previously (Wilkie *et al*., 1996). Briefly, cells were maintained, stimulated and extracted as for Western blotting. A 10 μl sample of homogenized cell extract was incubated for 20 min at 30°C in the presence of 15 μl of assay system comprising 25 mM $MgCl_2$, 1 mM substrate peptide APRTPGGRR and 50 μM ATP/[^{32}P]-ATP (1 μCi per tube). The reaction was terminated by the addition of 20% (w/v) trichloroacetic acid (20 μl per sample); each sample was blotted onto p81 paper and washed with 75 mM phosphoric acid, and counted for ^{32}P.

Cyclic AMP

Cyclic AMP was measured in trichloroacetic acid extracts of cells, after neutralization with ether extraction and addition of $NaHCO_3$ by use of the protein binding assay of Brown *et al*. (1971).

Materials

Cell culture supplies were from GIBCO (Paisley, Scotland) apart from foetal calf serum which was from Advanced Protein Products (West Midlands, U.K.). The antibody for tyrosine phosphorylated p44/p42 MAPK was from New England Biolabs. 2MeSATP was from Research Biochemicals (Semat Ltd, Herts, U.K.). Other reagents were from Fisher Scientific (Loughborough, U.K.) or from Sigma.

Data analysis was by GraphPad Prism2 (Graphpad Software Inc., San Diego, U.S.A.).

Results

Cytosolic Ca^{2+}

Figure 1a shows the effect of ATP on cytosolic Ca^{2+} levels of primary unpassaged cultures of brain capillary endothelial cells. Cells were loaded with Fura 2 and stimulated under perfusion conditions with 100 μM ATP. The responses of 5 individual cells from a single group of cells has been shown to

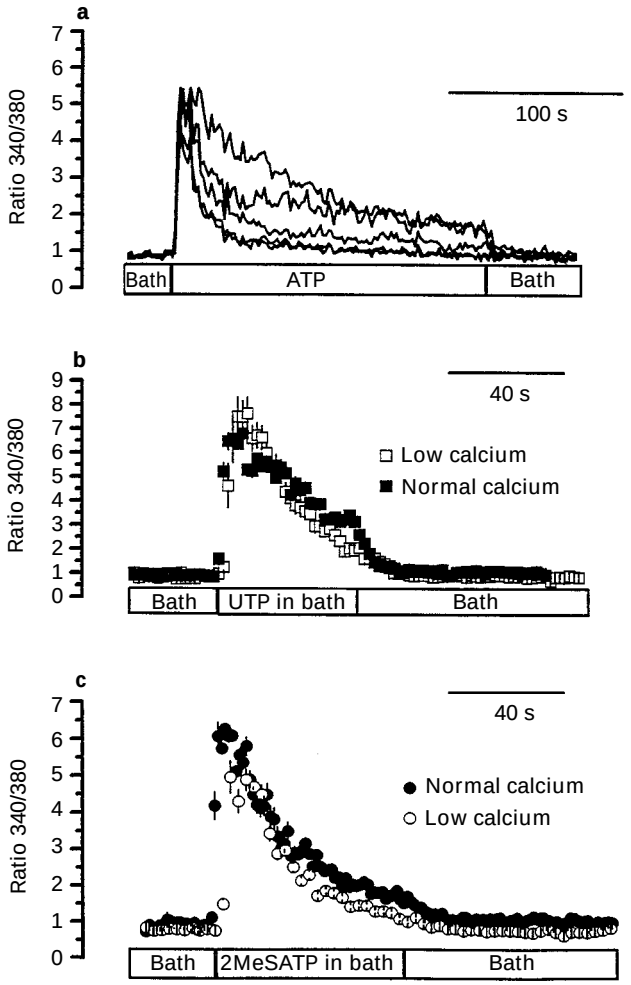

Figure 1 Time course of cytosolic Ca^{2+} responses to (a) ATP, (b) UTP and (c) 2MeSATP. Fura 2 loaded cells were perfused as indicated and the responses recorded as the 340/380 ratio from individual cells by microspectrofluorometry, as described in Methods. (a) Responses of 5 individual cells to 100 μM ATP; (b) responses to 300 μM UTP and (c) responses to 30 μM 2MeSATP in the presence of 2.5 mM extracellular Ca^{2+} or of no added Ca^{2+} and 2 nM EGTA. In (b) and (c) data presented are mean from 11–14 cells; vertical lines show s.e.mean.

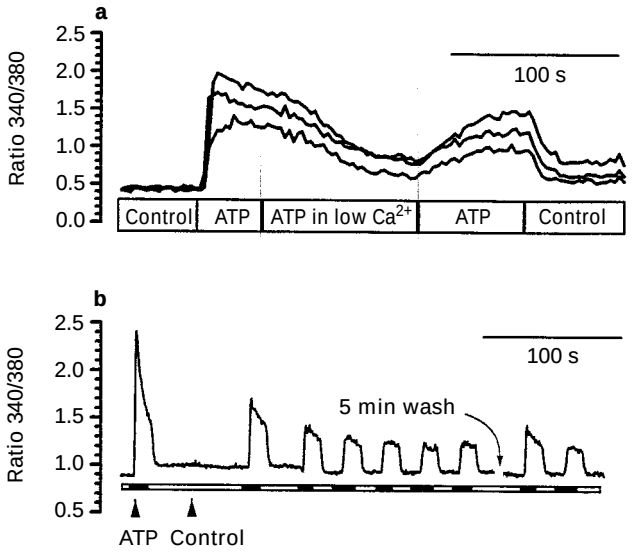

Figure 2 Time course of cytosolic Ca^{2+} response to ATP. (a) The response of 3 individual cells to ATP (100 μM) under conditions of 2.5 mM Ca^{2+}, followed by a period of no added Ca^{2+}/2 mM EGTA, followed by restoration of Ca^{2+} to 2.5 mM, in the continued presence of ATP. (b) The effect of repeated pulses of 100 μM ATP on the cytosolic Ca^{2+} response; the break in the trace represents a 5 min recovery period.

indicate the variations in response. Each cell gave a rapid rise to a peak within a few seconds, followed by a slower, and rather variable, decline towards a basal Ca^{2+} level, with a sustained plateau observed in 87% of cells studied. In most cases Ca^{2+} was still above basal with 2 min exposure to ATP, and decreased to basal upon removal of the agonist (Figure 1a). These cells also responded to other nucleotide agonists. Essentially all cells (99.5%; 1250 out of 1256) responded to ATP (100 μM), while most responded to 100 μM ADP (83.7%; 82 out of 98), 100 μM UTP (71.1%; 96 out of 135) and 30 μM 2MeSATP (70.1%; 192 out of 274). There was no significant difference in the size of responses seen with maximally effective concentrations of these different agonists. Responses to UTP and 2MeSATP are shown in Figure 1b and 1c, in both the presence and absence of extracellular Ca^{2+}. Peak responses were largely unchanged by the absence of extracellular Ca^{2+}, but there was a significant decrease in the level measured at 50 s after agonist addition ($P<0.0001$, Mann-Whitney U-test). This suggested that the peak was caused by mobilization of intracellular stores, followed by a contribution from influx of extracellular Ca^{2+}.

A Ca^{2+} entry phase was confirmed with further experiments with ATP as an agonist. When extracellular Ca^{2+} was removed while ATP was continued as a stimulant there was an accelerated decline towards basal (Figure 2a). Importantly, on restoring extracellular Ca^{2+} in the continued presence of ATP there was a substantial rise in intracellular Ca^{2+}. When this procedure was carried out in the absence of ATP there was no rise in intracellular Ca^{2+} on restoration of Ca^{2+} in the perfusing medium (not shown).

Further evidence suggestive of two separate phases to the increase in intracellular calcium was obtained by repeated applications of ATP made in the presence of extracellular calcium. The peak response to the second and subsequent exposures to ATP was abolished, while the plateau remained, although at a somewhat reduced level (Figure 2b).

Exposure of the unpassaged cells to α,β-methylene ATP (α,βMeATP) failed to increase $[Ca^{2+}]_i$ in 373 cells tested; the same cells did give a robust ATP response (Figure 3a). However, because there have been previous reports, in immortalized cells, of responses to α,βMeATP (Nobles *et al*., 1995), we took cells through a single passage and challenged them with this P2X agonist. We found that after a single passage the cells did give a Ca^{2+} response to 100 μM α,β-MeATP (Figure 3b).

Characteristically this was smaller than the response to ATP. In other respects the single passage cells gave a similar pattern of response to ATP and 2MeSATP as did unpassaged cells (data not shown). In all other experiments described here unpassaged cells were used.

Inositol (poly)phosphates

Figure 4 shows the $[^3H]$-$InsP_x$ response to increasing concentrations of ATP, UTP, 2MeSATP and ADP. The log EC_{50} values were: ATP, -5.1 ± 0.2 ($n=11$); UTP, -5.2 ± 0.2 ($n=6$); UDP, -5.3 ± 0.06 ($n=3$); ADP, -4.5 ± 0.7 ($n=4$) (mean$\pm$ s.e.mean, n=number of separate experiments, each in triplicate). 2MeSATP and α,βMeATP failed to generate a measurable response at concentrations (30 μM 2MeSATP and 100 mM α,βMeATP) which are maximal with respect to other responses. The results gave a rank order of agonist potency of ATP = UTP = UDP > ADP > > 2MeSATP = α,βMeATP. ATP, UTP and UDP all produced similar maximal responses, while ADP produced a lower maximal response: expressed as

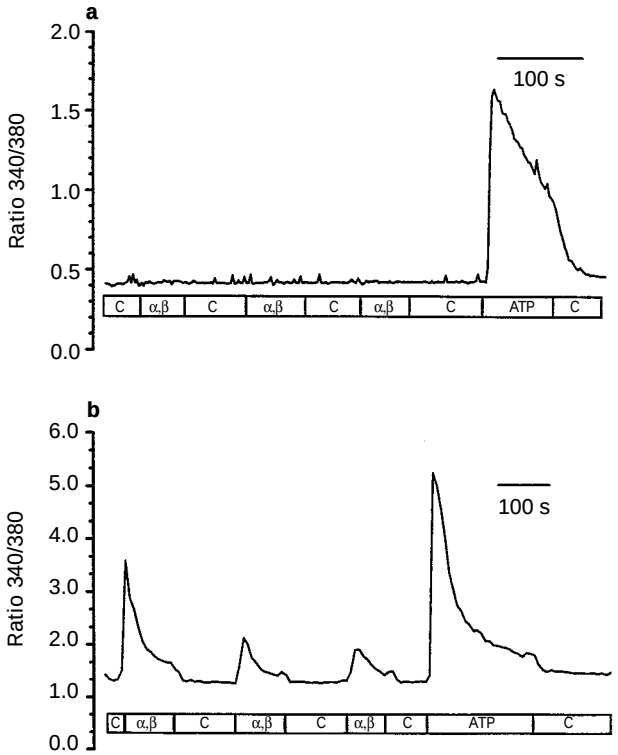

Figure 3 Stimulation with α,β-methylene ATP (α,β). In (a) the cytosolic Ca^{2+} response of unpassaged cells is shown, while in (b) the cells were used after a single passage. The concentrations of α,βMeATP and ATP were 100 μM. The mean response of 25 (a) and 30 (b) cells is shown. C, control.

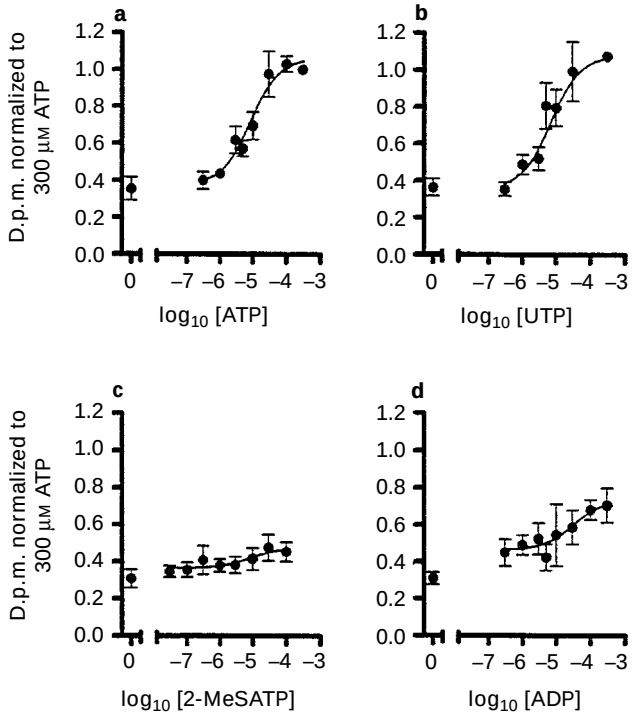

Figure 4 [^{3}H]-InsP_x responses to increasing concentrations of ATP (a), UTP (b), 2MeSATP (c) and ADP (d). Labelled cells were stimulated in the presence of 10 mM LiCl for 16 min with the agonists at concentrations indicated. The data for each experiment were normalized to the response to 300 μM ATP and data pooled from 3–7 experiments (each in triplicate) and presented as mean with vertical lines showing s.e.mean.

fold over basal, ATP = 3.1 ± 0.4 (n = 14), ADP = 2.2 ± 0.2 (n = 6), (ADP significantly different from ATP, $P < 0.001$ by Student's t test.

The results with 2MeSATP, consistent with earlier findings (Frelin *et al*., 1993; Vigne *et al*., 1994), show the presence of Ca^{2+} mobilization responses in the absence of detectable [^{3}H]-InsP_x elevations; this raises the possibility that this $P2Y_1$ agonist stimulated Ca^{2+} mobilization independently of agonist stimulated inositol (poly)phosphate generation. However, this approach cannot exclude the possibility of agonist stimulated formation of Ins(1,4,5)P_3 in the first seconds of stimulation. We used an assay for Ins(1,4,5)P_3 mass in cell extracts, when it is possible to measure responses at very short incubation times. Figure 5 shows that ATP and UTP (100 and 300 μM, respectively) both gave significant stimulations with 10 s incubations, with a subsequent decline to a plateau sustained for 5 min. In contrast, 30 μM 2MeSATP produced no detectable stimulation at any time investigated (10–300 s).

It has been found that UDP may act on cell surface receptors after conversion to UTP in the extracellular compartment by dinucleotide kinase activity (Harden *et al*., 1997). UTP formed by this route, or originating as a contaminant in the UDP, can be removed by hexokinase treatment, as described in Methods. In these experiments, in which [^{3}H]-InsP_x accumulation was measured, the concentration-response curve to UDP and ADP was unaffected by hexokinase treatment (not shown, 2 experiments each done in triplicate).

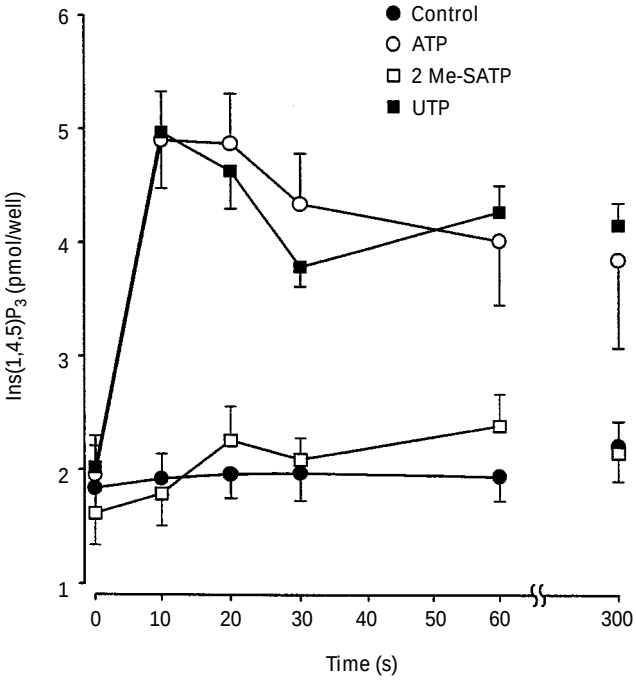

Figure 5 Accumulation of Ins(1,4,5)P_3 on stimulation with ATP, 2MeSATP, UTP or no agonist for the times shown. Cell extracts were then assayed for mass of Ins(1,4,5)P_3. Results are mean of a minimum of 4 separate experiments each in triplicate; vertical lines show s.e.mean.

Levels of cyclic AMP

To investigate whether the nucleotides alter levels of cyclic AMP in these cells we stimulated RBECs with the agonists under basal conditions, or when cyclic AMP levels were elevated with 5 μM forskolin. Table 1 shows that 2MeSATP (30 μM) had little or no effect on basal cyclic AMP levels, and no effect when applied with a concentration of forskolin which itself caused a modest increase in cyclic AMP levels. Further experiments with 30 μM 2MeSATP and forskolin at 0.1–20 μM, or with 5 μM forskolin and 2MeSATP at 0.1–100 μM

Table 1 Effect of ATP and 2MeSATP on cyclic AMP levels

	Basal	*ATP*	*Basal*	*2MeSATP*
Basal	0.71±0.17	1.17±0.31	1.23±0.09	1.31±0.12
Forskolin	7.14±2.81	26.7±7.6	4.28±0.61	4.59±2.26

Forskolin was at 5 μM, ATP at 300 μM and 2MeSATP was at 30 μM, each with 10 min incubation times. Data are pmol/well, mean±s.e.mean, $n=4$, from a single experiment. Data pooled across experiments are presented in the text.

also showed no effect of the $P2Y_1$ agonist on cyclic AMP levels (data not shown). When ATP was used as the agonist there was again no effect on basal cyclic AMP, but there was a substantial increase in the presence of 5 μM forskolin (Table 1). Data pooled across 4 experiments were: basal, 0.83±0.06; forskolin, 5.96±1.58; ATP, 1.22±0.06; ATP±forskolin, 26.44±11.47 (pmol per well). This illustrates some variation across experiments in the size of response to ATP and forskolin together. Despite this variation, the response to ATP plus forskolin was significantly higher than the response to forskolin alone (paired t test, $P<0.05$). In the presence of 5 μM forskolin ATPγS, an analogue of ATP relatively resistant to ectonucleotidase breakdown, gave a similar level of cyclic AMP elevation to that achieved by ATP (data not shown).

Activation of MAPK

Tyrosine phosphorylation of p42 and p44 MAPK was monitored by Western blot with an antiserum specific for the tyrosine phosphorylated state of these two forms of MAPK. Figure 6a shows that, under conditions in which there was no detectable phosphorylation in the absence of agonists, both ATP and UTP (100 μM) stimulation of cells resulted in strong phosphorylation bands for both p42 and p44 forms. 2MeSATP 30 μM also stimulated p42 and p44 MAPK phosphorylation. But as seen in Figure 6a, and reproduced over several experiments, the response to 2MeSATP was consistently weaker than the response to UTP. Over 3 such experiments densitometric scanning showed that the 2MeSATP response was 39.1±13.5% of the UTP response. Figure 6b shows the results of experiments with a peptide kinases assay for MAPK activity. The response to UTP gave a higher maximal and a more potent stimulation than 2MeSATP.

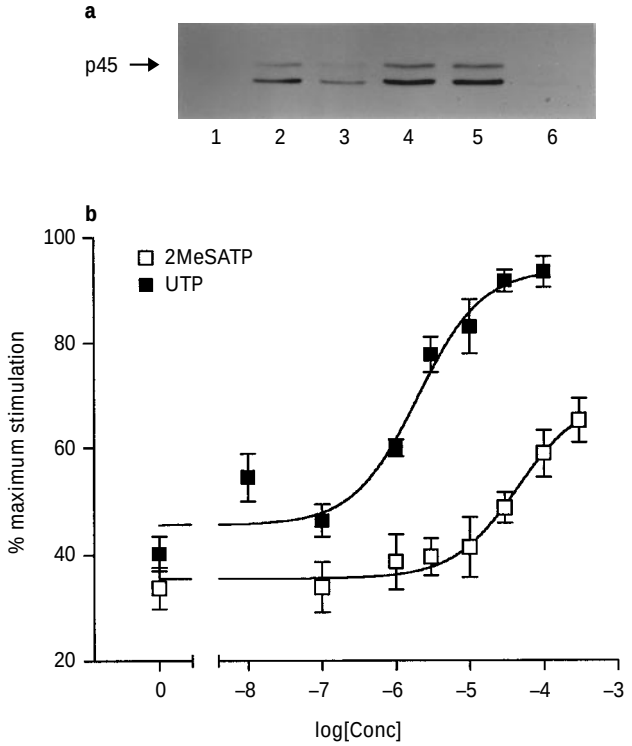

Figure 6 MAPK activation in response to nucleotide agonists. (a) p42 and p44 MAPK activation as shown by Western blot. Lane 1, control; lane 2, 100 μM ATP; lane 3, 30 μM 2MeSATP; lane 4, 100 μM UTP; lane 5, 100 μM ATPγS. Representative of 5 similar experiments. (b) Peptide kinase assay for MAPK activity. Concentration-response curves for 2MeSATP and UTP. Results are mean for 2 experiments each in triplicate; vertical lines show s.e.mean.

Discussion

Extensive studies have investigated the regulation of endothelial cells from major blood vessels by P2Y receptors; much of this work concentrated on the production of prostacyclin upon stimulation with ATP/ADP (Pearson *et al*., 1983) and the establishment of the view that this response was driven by raised cyctosolic Ca^{2+}, a consequence of phospholipase C (PLC) activation and Ca^{2+} mobilization from intracellular stores and Ca^{2+} entry (e.g. Needham *et al*., 1987; Carter *et al*., 1988; Lustig *et al*., 1992). Subsequent studies showed that the bovine aortic endothelial cell response is mediated by two coexisting receptors ($P2Y_1$ and $P2Y_2$) both linked to PLC (Wilkinson *et al*., 1993; Motte *et al*., 1993). Furthermore, stimulated prostacyclin production cannot be adequately described as a consequence of raised intracellular Ca^{2+}. Protein kinase C, tyrosine kinases and p42 and p44 MAPK all play a central role in this short term response of endothelial cells to P2Y agonists (Bowden *et al*., 1995; Patel *et al*., 1996a,b) and to thrombin (Wheeler-Jones *et al*., 1996). It has become apparent that brain capillary endothelial cells are also regulated by multiple P2Y receptors, but their exact nature is unclear, and their mechanism of action in controlling the blood-brain barrier has been largely interpreted as a response to raised intracellular Ca^{2+} (Revest & Abbott, 1992). One potential source of confusion is the use of different cell preparations, from unpassaged primary cells prepared by various techniques and cultured under various conditions, through to the development of immortalized or continuous cell lines retaining special characteristics of brain microvascular endothelial cells. We chose to use primary unpassaged cells in culture grown from a highly purified preparation of capillaries.

Using a clonal line of rat brain endothelial cells which has lost the $P2Y_2$ response, Webb *et al*. (1996) described a $P2Y_1$-like response which they showed to be coupled to inhibition of adenylyl cyclase. They concluded that this brain endothelial receptor is the same as the cloned rat $P2Y_1$ receptor. In addition they concluded that this receptor is responsible for the $P2Y_1$-like response described in rat C6-2B glioma cells (also coupled to inhibition of adenylyl cyclase). They interpreted this as an example of differential linkage of one receptor in different native cells; in some cells it is coupled to inhibition of adenylyl cyclase, in others to phospholipase C. This is apparently at variance with the interpretation of Boyer *et al*. (1994) and Schachter *et al*. (1996). Based on differential sensitivity of the receptors to antagonists and the transfection of the human $P2Y_1$ receptor into rat C6 glioma cells, they concluded that the PLC-linked and negative adenylyl cyclase-linked receptors are different. Direct comparison between the results of Webb *et al*. (1996) and our experiments is complicated by the very different nature of the cells used, but we have shown that on incubation with ATP (or hydrolysis resistant ATPγS) there is a substantial increase in the cyclic AMP levels over and above the level achieved by 5 μM forskolin alone. While the receptor and regulatory pathway responsible for this response are unknown, these results point to the possibility of a P2Y receptor linked, directly or indirectly, to an increase in cyclic AMP.

Under the conditions of our experiments 2MeSATP had no influence over either basal or forskolin (5 μM) elevated cyclic AMP levels in unpassaged primary cultures of rat brain en-

dothelial cells. We conclude from this that there is not a $P2Y_1$ receptor coupled to changes in cyclic AMP levels in these primary rat brain capillary endothelial cell cultures. A further possibility to be considered is that the $P2Y_2$ receptor, coupled in the main to PLC activation, may also coupled to an adenylyl cyclase stimulating G protein within the same cells. Precedent for $P2Y_2$ receptors linked to two diverging G protein pathways has been described by Baltensperger and Porzig (1997) in human erythroleukaemia cells. This study demonstrated that $P2Y_2$ linked through G_{16} (a member of the G_q subfamily found only in haematopoietic cell types) caused intracellular Ca^{2+} mobilization. In addition, this $P2Y_2$ receptor, linked through a member of the G_i class in a non-calcium dependent manner, enhanced the calcium increase in response to prostaglandin E_1.

As stated above, the data presented here on cytosolic Ca^{2+} and inositol phosphates levels are in the main part consistent with the presence of a $P2Y_2$ receptor coupled to PLC (Frelin *et al*., 1993; Vigne *et al*., 1994; Nobles *et al*., 1995). The only contraindication is the failure of hexokinase treatment to attenuate the response to UDP. Nicholas *et al*., (1996) found that the response of the cloned and transfected human $P2Y_2$ receptor to UDP was attenuated by removal of UTP by hexokinase treatment, indicating that the apparent response to UDP is caused by UTP, either as a contaminant of UDP or formed by ecto-dinucleotide kinase on incubation of the cells with UDP. In studies run in parallel with the brain endothelial cells, we have confirmed that hexokinase attenuates the response to UDP of 1321N1 cells transfected with the human $P2Y_2$ receptor (S. Charlton and M.R. Boarder, unpublished results). However, we found here that there was no effect of hexokinase on the response of rat brain endothelial cells to UDP or ADP. The explanation for this divergence from the agonist profile predicted for a $P2Y_2$ receptor is unclear, but it may represent a species difference in the pharmacology of the $P2Y_2$ receptors, or a difference dependent on the cell type in which the receptor is studied.

We have shown that the Ca^{2+} response to ATP and UTP is accompanied by a PLC response, not only as measured in [^{3}H]-inositol labelled cells, but also as a rapid accumulation of $Ins(1,4,5)P_3$, with a peak which declines after 10 s. This coincides with the Ca^{2+} response to ATP and UTP, the characteristics of which are consistent with a rise to peak caused by intracellular Ca^{2+} mobilization and a contribution of extracellular Ca^{2+} entry to the plateau phase. The source of the decrease in calcium mobilization phase with repeated agonist application (Figure 2b) may represent a desensitization phenomenon at the level(s) of the P2-receptor or events downstream in the G protein signalling cascade. An alternative explanation may be an unexpectedly slow re-filling of the internal stores of calcium. Further studies will be required to address this issue. Of particular interest here is the observation that 2MeSATP generates a similar peak elevation in $[Ca^{2+}]_i$ and that this response is also largely independent of extracellular Ca^{2+}. This is significant because it is not accompanied by an elevation in the formation of [^{3}H]-$InsP_x$ in [^{3}H]-inositol labelled cells, as shown previously in passaged cells (Frelin *et al*., 1993; Vigne *et al*., 1994; Feolde *et al*., 1995). However, these studies did not exclude the possibility of transient formation of $Ins(1,4,5)P_3$ during the first few seconds or minute of stimulation. This is the period of the initial Ca^{2+} response. Such a short lived formation of the Ca^{2+}-mobilizing $Ins(1,4,5)P_3$ would not necessarily result in a detectable [^{3}H]-$InsP_x$ response, which is measured over a longer time course. We have shown here that while a rapid accumulation of $Ins(1,4,5)P_3$ is apparent with ATP or UTP stimulation, no such response occurs with 2MeSATP stimulation. This indicates that the Ca^{2+} mobilizing 2MeSATP response is likely to be independent of agonist stimulated increases in the level of $Ins(1,4,5)P_3$. This suggests the presence of another pathway from the G protein-coupled receptor to intracellular Ca^{2+} stores (e.g. Simpson *et al*., 1995).

These observations demonstrate the presence of two receptors which generate a similar rise in cytosolic Ca^{2+}. One responds to both ATP and UTP and is coupled to PLC, and has characteristics which are best interpreted as reflecting the presence of a $P2Y_2$ receptor. The other response is to 2MeSATP, an agonist selective for $P2Y_1$ and P2X receptors. The P2X receptors are intrinsic ion channel receptors; a Ca^{2+} response would be dependent on extracellular Ca^{2+}. In this study and in that of Vigne *et al*. (1994) it can be seen that the response to 2MeSATP is largely independent of extracellular Ca^{2+}, so we conclude that P2X receptors are not responsible. It appears then that the response is '$P2Y_1$-like', in that it is a 2MeSATP sensitive receptor coupled to Ca^{2+} mobilization, but without the phosphoinositide breakdown and $Ins(1,4,5)P_3$ formation seen with other $P2Y_1$ receptors coupled to Ca^{2+} mobilization.

If the regulation of brain endothelial cell function by these two different $[Ca^{2+}]_i$ coupled receptors is solely a consequence of events downstream of elevated Ca^{2+}, then we might interpret this situation as two receptors ($P2Y_2$ and $P2Y_1$-like) bringing about the same outcome in terms of cellular regulation, but by independent pathways. However, as outlined above, in other endothelial cells it has been shown that events other than Ca^{2+} control the cells short-term response, including the p42/p44 MAPK cascade. Various signalling pathways converge on MEK, the dual specificity kinase which both threonine- and tyrosine-phosphorylates p42 and p44 MAPK leading to the activation of kinase activity. The use of an immunoblot procedure, which is specific for the 204-phosphotyrosine form of MAPK, and the peptide kinase assay system, together provide an index of activation of the enzymes. Here we used these procedures to investigate whether this MAPK cascade may be involved in the response of brain capillary endothelial cells to activation of either the $P2Y_1$-like $Ins(1,4,5)P_3$- independent response or the PLC-coupled $P2Y_2$ response. Our results have shown that UTP, ATP, ATPγS and 2MeSATP all lead to MAPK activation, as shown by Western blot, and for UTP and 2MeSATP, by peptide kinase assay. The stimulation of MAPK activity by UTP had a potency consistent with a response at a $P2Y_2$ receptor, as indicated by comparison with other responses. However, the concentration-dependency of the 2MeSATP response suggested it was not likely to be due to activation of a $P2Y_1$ receptor. MAPK has been shown to be involved in endothelial secretory responses (Patel *et al*., 1996; Wheeler-Jones *et al*., 1996). Tyrosine kinase cascades (which includes MAPK) have shown to regulate endothelial permeability and the blood-brain-barrier (Staddon & Rubin, 1996). It seems likely, therefore, that while these two receptors produce similar Ca^{2+} responses, they will differ in their functional regulation of brain endothelial cells.

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