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Evidence that $P2Y_4$ nucleotide receptors are involved in the regulation of rat aortic smooth muscle cells by UTP and ATP

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1 Previous studies have shown that ATP and UTP are able to stimulate phospholipase C (PLC) and proliferation in cultured aortic smooth muscle cells. Here we set out to characterize the receptor responsible, and investigate a possible role for p42 and p44 mitogen activated protein kinase (MAPK) in the proliferative response.

2 The phospholipase C response of spontaneously hypertensive rat (SHR) derived aortic smooth muscle cells in culture showed that the response to ATP was partial compared to the response to UTP.

3 Further studies characterized the responses of the SHR derived cells. UTP was the only full agonist with the SHR cells; UDP gave a partial response while ADP, 2-methythio-ATP and α,β-methylene ATP were essentially ineffective. The response to UDP was almost lost in the presence of hexokinase, consistent with this being due to extracellular conversion to UTP. These observations are inconsistent with the response being mediated by either $P2Y_1$ or $P2Y_6$ receptors.

4 When increasing concentrations of ATP were present with a maximally effective concentration of UTP, the size of the response diminished, consistent with UTP and ATP acting at a single population of receptors for which ATP was a partial agonist. This is inconsistent with a response mainly at $P2Y_2$ receptors.

5 1321N1 cells transfected with human $P2Y_4$ receptors gave a similar agonist response profile, with ATP being partial compared to UTP, loss of response to UDP with hexokinase treatment, and with the response to UTP diminishing in the presence of increasing concentrations of ATP.

6 Use of the reverse transcriptase-polymerase chain reaction confirmed the presence of mRNA encoding $P2Y_4$ receptors in SHR derived vascular smooth muscle cells. Transcripts for $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors, but not $P2Y_1$ receptors, were detected.

7 Stimulation of SHR derived cells with UTP enhanced the tyrosine phosphorylation of both p42 and p44 MAPK, and the incorporation of [^{3}H]-thymidine into DNA. Both these responses were diminished in the presence of an inhibitor of activation of MAPK.

8 These results lead to the conclusion that in SHR derived cultured aortic smooth muscle cells, PLC responses to extracellular UTP and ATP are predominantly at $P2Y_4$ receptors, and suggest that these receptors are coupled to mitogenesis via p42/p44 MAPK.

Keywords: P2Y receptors; $P2Y_4$ receptors; vascular smooth muscle cells; mitogenesis; SHR; UTP

Introduction

P2 nucleotide receptors responding to extracellular adenosine 5′-triphosphate (ATP), ADP, uridine 5′-triphosphate (UTP) and UDP regulate diverse bodily functions by acting on families of ion channel P2X receptors or G protein-coupled seven transmembrane P2Y receptors (Boarder *et al*., 1995; Burnstock, 1996). Several subtypes of P2Y receptors have recently been cloned, and their pharmacology related to that of native receptors. In the vasculature P2Y receptors have been shown to regulate both vascular smooth muscle (VSM) cells and vascular endothelial cells. A principal source of extracellular nucleotides in vasculature is that released from activating platelets. In healthy vasculature the main influence of nucleotides released from this source is on the endothelium, where co-existing $P2Y_1$ and $P2Y_2$ receptors, coupled to phospholipase C (PLC) and cytosolic Ca^{2+}, stimulate the production of mediators such as prostacyclin and nitric oxide (Wilkinson *et al*., 1993; 1994; Motte *et al*., 1993). These have a relaxant and antiproliferative influence on VSM cells, and an antiplatelet action, attenuating further platelet activation and nucleotide release. However, in those vascular conditions characterized by a damaged endothelium, there is a deficit in the production of these endothelial derived mediators in response to platelet activation. As a result there is increased release of nucleotides from platelets, which have direct access to VSM cells, at a time when the inhibitory influence of the endothelium on VSM is diminished. Under these circumstances the principal influence of platelet-derived nucleotides may be to stimulate proliferation of VSM cells, contributing to hyperplasia, atherosclerotic plaque formation, stenosis and restenosis encountered in various clinical conditions. Supporting this possibility are observations that VSM cells respond to nucleotides by acting at P2Y receptors coupled to PLC and cytosolic Ca^{2+}. Responses to UTP have suggested the presence of a $P2Y_2$ receptor (Pffeilschifter, 1990; Kitajima *et al*., 1994; Pacaud *et al*., 1995; Miyagi *et al*., 1996). However, many of the results do not distinguish between the various receptors (currently $P2Y_2$–$P2Y_6$) which respond to the pyrimidines UTP and UDP, and some data are apparently inconsistent with a response solely at a $P2Y_2$ receptor (Garcia-Velasco *et al*., 1995).

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There are also reports of nucleotide stimulation of mitogenesis in VSM cells, either alone or as a co-mitogen (Wang *et al*., 1992; Malam-Souley *et al*., 1993; Erlinge *et al*., 1995); again the nature of the receptor is unclear. A recent study has implicated the Raf-mitogen activated protein kinase (MAPK) cascade in this proliferative response (Yu *et al*., 1996). This is consistent with extensive investigation of the role of this cascade in the response of VSM cells to activation of AT_1 receptors, another example of G protein-coupled mitogenic responses (reviewed in Berk & Corson, 1997). Further interest in P2Y responses of VSM cells was engendered by the report of cloning of a novel P2Y receptor ($P2Y_6$) from rat aorta cDNA which shows selectivity for pyrimidines (Chang *et al*., 1995). It has been suggested that this receptor may be responsible for the PLC and proliferative response of VSM cells (Chang *et al*., 1995).

We have previously used the comparison of cultured VSM cells from spontaneously hypertensive rats (SHR) and normotensive controls (WKY) to investigate the responses to angiotensin II (Morton *et al*., 1995; Baines *et al*., 1996; Wilkie *et al*., 1996; 1997). The SHR, but not WKY, derived cells gave a mitogenic response to the addition of angiotensin II alone, relating to a greater stimulation of PLC, PLD, Ca^{2+} and p42 and p44 MAPK. Here we present, for the first time, evidence for a role for $P2Y_4$ receptors in regulation of VSM. We show that mRNA for a number of P2Y receptors, including $P2Y_4$ is expressed in the SHR derived cells, and that nucleotide agonists stimulate a PLC response with a pattern consistent with a response predominantly at $P2Y_4$ receptors.

Methods

Cell preparation and culture

VSM cells were prepared as described by Davies *et al*. (1991). After the arterial pressure of 12 week old SHR and WKY rats had been determined, the thoracic aorta was removed, stripped of adventitia and enzymatically digested. Clonal cultures were established and after 5 days those colonies with smooth muscle cell morphology were combined, and cultured in Dulbecco's modified Eagle's medium supplemented with 10% foetal calf serum, penicillin (100 iu ml^{-1}), streptomycin (100 $\mu g\ ml^{-1}$) and glutamine (27 mg ml^{-1}) in 175 cm^2 flask at 37°C in 5% CO_2, 95% air. Cells were used for experiments between passages 6 and 12, seeded into 24 well plates for [^{3}H]-$InsP_x$ and [^{3}H]-thymidine assays and 80 cm^2 flasks for phospho-MAPK Western blots, and maintained serum free for 24 h before use. Cells showed 100% positive smooth muscle actin immunofluorescence. The human $P2Y_4$ receptor transfected 1321N1 cells were used as described previously (Charlton *et al*., 1996).

RT–PCR and analysis methods

Total RNA was prepared from $\sim 4 \times 10^6$ cultured VSM cells ($n = 4$) using a micro RNA isolation kit (Stratagene), resuspended in 100 μl H_2O, treated with 200 units deoxyribonuclease I (Gibco BRL) for 15 min at 37°C, phenol/chloroform and chloroform extracted and isopropanol precipitated. cDNA was synthesized from 5 μg of each total RNA preparation in a 40 μl reaction volume, in the presence of 2 μg of the primer 5′-TTTTTTTTTTTTTTTT(G/A/C)-3′, 2 mM dNTPs, 10 units ribonuclease inhibitor (Gibco BRL) and 400 units of Superscript II reverse transcriptase (Gibco BRL) according to the manufacturer's recommendations.

Oligonucleotide amplification primers (24-mers) were designed from the rat $P2Y_1$ (Tokuyama *et al*., 1995), $P2Y_2$ (Chen *et al*., 1996), $P2Y_4$ (Webb *et al*., 1998) and $P2Y_6$ (Chang *et al*., 1995) sequences. $P2Y_1$: sense primer 5′-TGGCGTGGTGCTGCACCCTCTCAAGTC-3′, antisense primer 5′-CGGGACAGTCTCCTTCTGAATGTA-3′; $P2Y_2$: sense primer 5′-CTGCCAGGCACCCGTGCTCTACTT-3′, antisense primer 5′-CTGAGGTCAAGTGATCGGAAGGAG-3′; $P2Y_4$; sense primer 5′-CACCGATACCTGGGTATCTGCCAC-3′, antisense primer 5′-CAGACAGCAAAGACAGTCAGCACC-3′; $P2Y_6$; sense primer 5′-GGAGACCTTGCCTGCCGCCTGGTA-3′, antisense primer 5′-TACCACGACAGCCATACGGGCCGC-3′.

RT–PCR was performed in buffer containing 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25°C), 0.01% Tween-20, 1.5 mM $MgCl_2$ using 3 μl of the reverse transcription, 200 ng of each primer, 200 μM of each dNTP and 2.5 units of Biotaq polymerase (Bioline) in a total volume of 50 μl under the following conditions: 60 s at 94°C, 30 s at 65°C, 60 s at 72°C for 30 cycles followed by 1 cycle at 72°C for 5 min. PCR reactions that included a mock cDNA synthesis reaction, where no reverse transcriptase was added, were set up in parallel as a control for genomic DNA contamination in the RNA sample and for contaminating DNA in the PCR reaction. Two independent PCR reactions were carried out for each cDNA synthesis. Amplification products (10 μl) were resolved on a 2% (w/v) agarose gel by electrophoresis. The bands were excised from the gel, cloned into the pCRIII vector (TA cloning kit, Invitrogen) and sequenced to confirm their identity.

Total [^{3}H]-inositol (poly)phosphates

Cells at 80–90% confluence in 24-well multiwells were labelled for 24 h with *myo*-[2-^{3}H]-inositol (0.037 Mbq ml^{-1}, 0.5 ml per well) in Medium M199 with 25 iu ml^{-1} penicillin, 25 $\mu g\ ml^{-1}$ streptomycin, at 37° in 5% CO_2. Stimulations were for 15 min in the presence of 10 mM LiCl, followed by extraction of total [^{3}H]-$InsP_x$ on small Dowex-1 (Cl^-) columns.

Incorporation of [^{3}H]-thymidine

Cells at 80% confluence were maintained serum free for 24 h and then exposed to agonist at the concentration indicated, in a serum free medium, for 20 h. [^{3}H]-thymidine (0.074 MBq ml^{-1}) was then added followed by a further 4 h incubation. The medium was then aspirated and the cells washed twice with balanced salt solution (BSS, mM: NaCl 125, KCl 5.4, $NaHCO_3$ 16.2, HEPES 30, NaH_2PO_4 1, $MgSO_4$ 0.8, $CaCl_2$ 1.8, glucose 5.5; buffered to pH 7.4 with NaOH and gassed with 95% O_2/5% CO_2). The cell monolayer was then placed on ice and washed sequentially with ice cold 5% trichloroacetic acid and ethanol, taken up into 0.1 M NaOH and scintillation counted.

Phospho-MAPK Western blots

Cells at 90% confluence, maintained serum free for 24 h, were washed twice with BSS for 10 min. When PD 98059 was used it was added at this time, and the cells left for a 20 min preincubation period, following which they were stimulated with agonists for 5 min. Stimulations were terminated by snap freezing the cells with liquid nitrogen and the cells were lysed in homogenizing buffer (20 mM Tris pH 7.4, 2 mM EDTA,

10 μg ml^{-1} leupeptin, 20 μM E-64 (trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane), 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 phenylmethylesulphonyl fluoride and 0.1% Triton X-100), scraped, bath sonicated for 10 min and spun at 14 000 *g* for 10 min at 4°C. Supernatants were then used immediately for immunoblots with a phospho-specific MAPK antibody (New England Bioproducts Ltd.) raised against a synthetic phospho-tyrosine peptide corresponding to residues 196 to 209 of human p44^{mapk} (DHTGFLTEY(p)VATRWC). This recognizes p44^{mapk} and p42^{mapk} only when phosphorylated at Tyr204. Detection was with the Amersham Enhanced Chemiluminescence (ECL) kit. The use of this Western blot procedure to measure phosphorylation of p42 and p44 MAPK as an index of MAPK activation by its upstream kinase was as described and validated in Wilkie *et al.* (1996) and Patel *et al.* (1996).

Materials

Cell culture medium was purchased from GIBCO (Paisley, U.K.) The horseradish peroxidase conjugated secondary antibody and ECL reagents were purchased from Amersham (Bucks, U.K.), as were *myo* [2-^{3}H]-inositol and [methyl-^{3}H]-thymidine. Antibodies to phosphotyrosine phosphorylated MAPK were from New England Bioproducts (Herts, U.K.). All other chemicals and reagents were from Sigma (Poole, U.K.).

Statistics

Statistical analyses were by analysis of variance followed by Dunnet's *post hoc* test.

Results

PCR was applied to template cDNA synthesized from RNA extracted from cultured SHR derived VSM cells using primer pairs designed to amplify regions of the cDNAs encoding the rat $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors (Figure 1). Amplification products were detected for the $P2Y_2$, $P2Y_4$ and $P2Y_6$, while amplification of $P2Y_1$ was not apparent. All three products were cloned and sequenced to confirm their identity, which in all cases authenticated the products.

The [^{3}H]-InsP_x responses to UTP and ATP of [^{3}H]-inositol labelled aortic smooth muscle cells of both SHR and WKY origin are shown in Figure 2. The data show that for both agonists the maximal responses of the SHR derived cells were greater than those of the WKY derived cells, with the difference being greatest for responses to UTP (difference between concentration-response curves for SHR and WKY when stimulated by UTP was significant at $P<0.05$). The position of the concentration-response curves was essentially the same for each cell type.

The results in Figure 2 also show that in the SHR derived cells the maximal response to UTP was higher than that to ATP ($P<0.05$), while in the WKY derived cells the responses to the two agonists were the same. Further experiments were undertaken to characterize the response in the SHR derived cells. Figure 3 shows concentration-response curves to further agonists. Again ATP elicited a smaller maximal response than UTP. ADP elicited only a small response even at the largest concentrations used, and no response was seen to α,β-methylene-ATP or to 2-methylthio-ATP. In further experiments we found that ATPγS gave the same response as ATP at

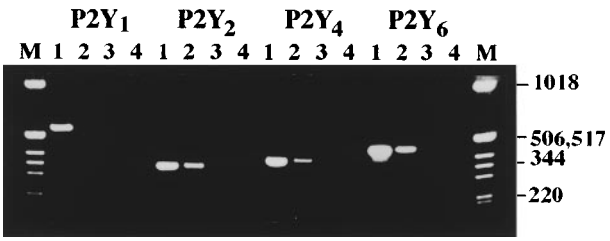

Figure 1 Agarose gel electrophoresis of PCR amplification products from VSM cells, (M), size markers: 1 Kb ladder (Gibco BRL), appropriate sizes are indicated. Primers included in the amplifications are indicated above the lanes. For each receptor amplification lane 1 is a PCR reaction from 5 ng of plasmid DNA containing the whole coding region of the appropriate P2Y receptor, lanes 2 and 3 incorporated cDNA syntheses where reverse transcriptase was included or excluded respectively, while no exogeneous template was added to lane 4. The figure is representative of four independent experiments. For further details refer to the Methods section.

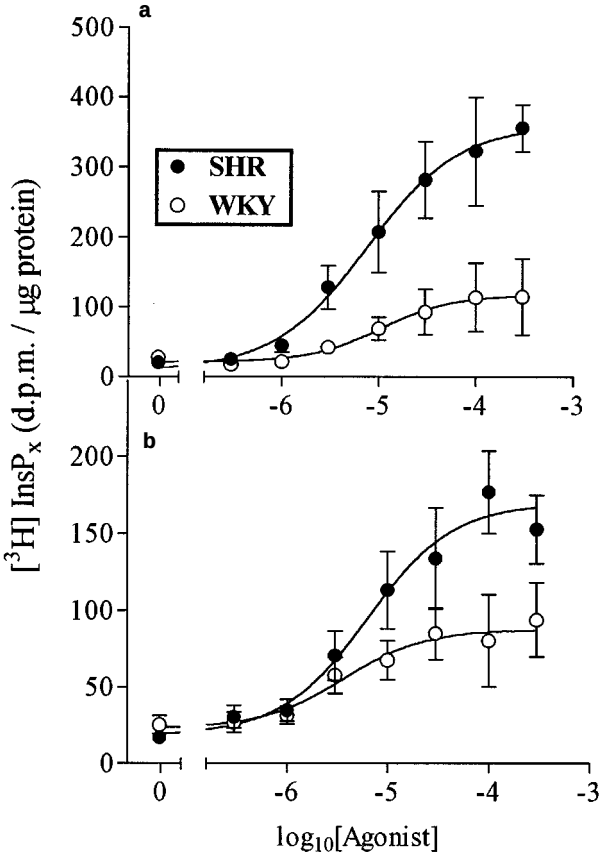

Figure 2 [^{3}H]-inositol (poly)phosphates response of SHR and WKY VSM cells to (a) UTP and (b) ATP. Cells were preincubated in 10 mM LiCl for 10 min and incubated with agonist and 10 mM LiCl for 15 min. The experiment was undertaken in triplicate with data expressed as mean of 3 separate experiments; vertical lines show s.e.mean.

300 μM, while there was no response to adenosine at 300 μM (data not shown). UDP also gave a response in these SHR derived cells (Figure 4). At the maximal concentration used this response was 63.6±26.4% of that of UTP at the same concentration. It has recently been shown that responses to UDP added to cells in culture may be caused by the conversion of UDP to UTP by the action of extracellular nucleoside diphosphokinase activity, as well as the presence of UTP

contaminating the supplies of UDP (Nicholas *et al*., 1996; Harden *et al*., 1997). The same studies described the use of hexokinase in the presence of glucose to remove any UTP present. Experiments presented in Figure 4 show that with hexokinase treatment the majority of the response to UDP was lost, apart from at the highest concentration used.

Certain antagonists of P2 receptors show some ability to discriminate between different P2Y receptors. We have examined the effect of the presence of suramin, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS) and 8,8′-(carboxylbis (imino-3,1-phenylene)) bis -1,3,5-napthalentrisulfonic acid (NF023) on the [^{3}H]-InsP$_x$ response of the SHR derived cells to stimulation with UTP. Neither PPADS nor NF023 had any effect on the response at concentrations from 1–300 μM with 300 μM UTP, or with antagonists at 300 μM and concentrations of UTP from 0.3–300 μM (data not shown). However, suramin did attenuate the response to UTP in a concentration (30–300 μM)-dependent manner (Figure 5). There was no significant effect on EC$_{50}$. However, there was a significant reduction in the response to the maximum concentration of UTP used to 75.7±1.79%, (100 μM suramin), and 67.5±6.8%, (300 μM suramin) of the response seen with UTP alone ($P<0.01$ and $P<0.05$, respectively).

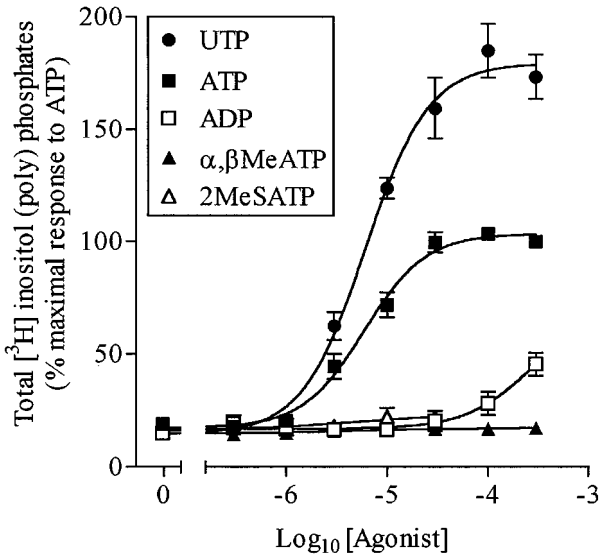

Figure 3 [^{3}H]-inositol (poly)phosphates response of SHR VSM cells to UTP, ATP, ADP, α,β-MeATP and 2MeSATP. Cells were preincubated in 10 mM LiCl for 10 min and incubated with agonist and 10 mM LiCl for 15 min. Each point represents the mean percentage maximal response to ATP from three experiments, each performed in triplicate, except for α,β-MeSATP which is the mean of two experiments performed in triplicate. Vertical lines show s.e.mean.

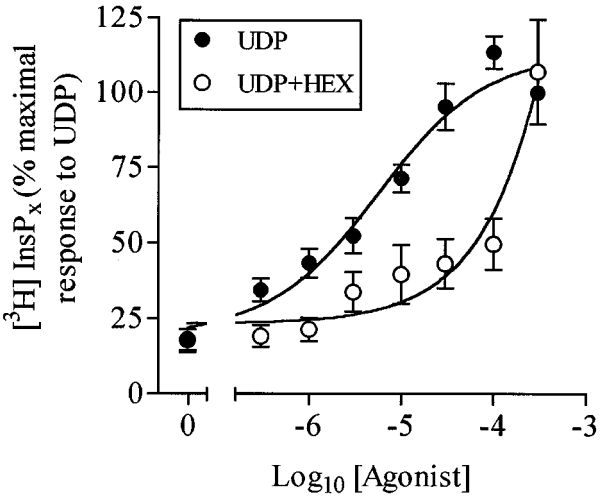

Figure 4 [^{3}H]-inositol (poly)phosphates response of SHR VSM cells to UDP in the absence and presence of hexokinase and glucose. Stock solutions of UDP (5 mM) were incubated for 1 h with hexokinase (50 units ml^{-1}) and glucose (110 mM). SHR cells were preincubated in hexokinase (1 unit ml^{-1}), glucose (22 mM) and LiCl (10 mM); or with LiCl (10 mM) alone (controls) for 10 min. Cells were then incubated in hexokinase-treated UDP and untreated UDP with 10 mmol l^{-1} LiCl for 15 min. Each point represents the mean percentage maximal response from three different experiments, each performed in triplicate.

The lower response to ATP compared to UTP seen in the SHR derived cells could be due to UTP being a full agonist but ATP acting as a partial agonist at the same receptor, or due to responses at some combination of receptors. To provide information relating to this issue we looked at the effect of stimulating with UTP and ATP simultaneously. If the responses of the two agonists were at separate receptors we would anticipate an additive effect. The results, presented in Figure 6, show that in contrast to this, the effect of ATP is to reduce the stimulation in response to UTP, using a concentration of UTP which was just sufficient to give a maximal response. The effect of 30 μM to 300 μM ATP was significant by analysis of variance with the effect of 100 μM and 300 μM ATP significant at $P<0.05$ by Dunnet's *post hoc* test. These results are consistent with a single receptor at which ATP is a partial agonist (see Discussion).

Two cloned P2Y receptors have been shown to have both ATP and UTP as effective agonists. One is the P2Y$_2$ receptor, for which both UTP and ATP are full agonists. The other is P2Y$_4$, for which ATP has been found to be a partial agonist (Communi *et al*., 1995). We therefore directly compared the results from the VSM cells with those from 1321N1 cells transfected with the human P2Y$_4$ receptor. Figure 7a shows that ATP gives a response which is partial compared to UTP, while UDP also gives a partial response which is essentially lost on hexokinase treatment (Figure 7b). 2MeSATP and ADP gave essentially no response. These results are similar to those described above for the VSM cells. The EC$_{50}$ for ATP was essentially the same for the P2Y$_4$ transfectants and the VSM cells. In the P2Y$_4$ transfectants the EC$_{50}$ for UTP was shifted to the left of the EC$_{50}$ for ATP, and to the left of the EC$_{50}$ for UTP in the VSM cells (see Discussion). To compare the

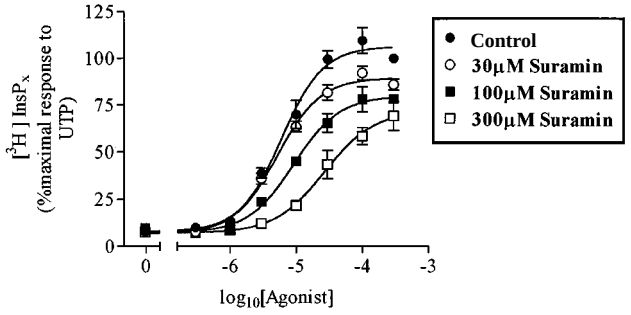

Figure 5 The effect of 30, 100 and 300 μM suramin on dose-response curves to UTP in SHR VSMC. Cells were preincubated with 10 mM LiCl (control) or with 30 μM, 100 μM or 300 μM suramin and 10 mM LiCl for 10 min. Incubations with UTP were for 15 min. Each point represents the mean percentage maximal response from three different experiments, each performed in triplicate. Vertical lines show s.e.mean.

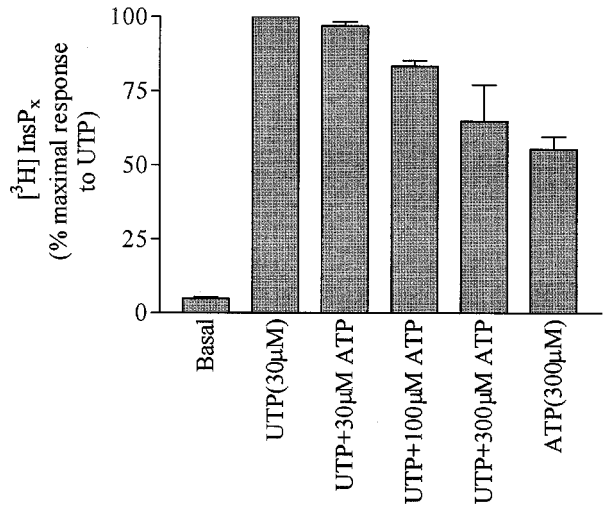

Figure 6 The effect of ATP on the [^{3}H]-inositol phosphate response to UTP on SHR cells. Cells were preincubated with 10 mM LiCl for 10 min and stimulated with UTP (30 μM), ATP (300 μM), or UTP (30 μM) plus ATP (30, 100 and 300 μM) for 15 min. Each column represents the mean$\pm$s.e.mean percentage from three different experiments, each performed in triplicate.

antagonist aspects of ATP action at the two cell types, the effect of the presence of ATP on the response of P2Y$_4$ transfectants to UTP was investigated using essentially the same experimental design as that for the VSM cells. A concentration of UTP which just gave a maximum response was used. The results in Figure 8 confirm that ATP acts as an antagonist at the P2Y$_4$ receptor, with similar partial agonist characteristics as those described above for the SHR derived VSM cells.

To determine whether these [^{3}H]-InsP$_x$ responses to ATP and UTP are accompanied by stimulation of mitogenesis, we investigated the incorporation of [^{3}H]-thymidine into DNA. We found that 300 μM ATP and UTP gave stimulations of 2.9$\pm$0.7 and 3.1$\pm$1.1 fold respectively (from 5 experiments each in triplicate). For these SHR derived cells the relative stimulation by UTP compared to ATP in each individual experiment was variable, and with WKY cells no consistent stimulation of [^{3}H]-thymidine incorporation was apparent. In previous work we found significance of stimulation of p42/p44 MAPK in the mitogenic response of SHR cells to angiotensin II (Wilkie *et al*., 1996). Here, we show that UTP stimulates the tyrosine phosphorylation of p42 and p44 MAPK in the SHR derived cells (Figure 9a). The phosphorylated p42 band was characteristically stronger than the phosphorylated p44 band, as in previous studies (Wilkie *et al*., 1996; Patel *et al*., 1996). Over three experiments densitometric analysis of immunoblots showed that there was an increase of 3.4$\pm$0.12 fold over basal of the phospho-MAPK immunoreactivity corresponding to the p42 band. This stimulation of an index of MAPK activation by UTP was effectively prevented by the inhibitor of the upstream MAPK kinase (MEK), PD 98059 (Figure 9a). Over 3 such experiments, the UTP (300 μM) stimulated phosphorylation of the p42 band was reduced by 10 and 30 μM PD 98059 to 9.1$\pm$3.6% and 8.6$\pm$1.6%, respectively. The phosphorylations of the p44 band were similarly reduced by 10 μM and 30 μM PD 98059 to 12.0$\pm$4.2% and 12.1$\pm$5.8% respectively. Figure 9b shows that PD 98059 also attenuated the UTP stimulation of [^{3}H]-thymidine incorporation. Pooled across 3 experiments the effect of PD 98059 on UTP stimulated mitogenesis was significant by analysis of variance and, for example, at $P<0.01$ for 10 μM PD 98059 by Dunnet's *post hoc* test.

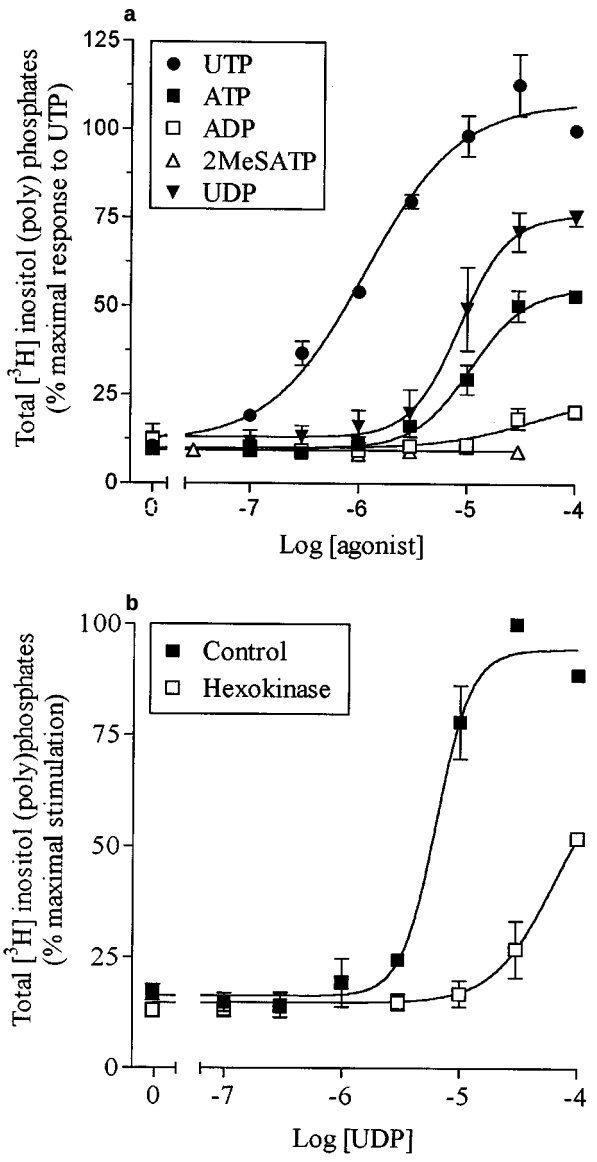

Figure 7 (a) [^{3}H]-inositol (poly)phosphates response of P2Y$_4$ transfected 1321N1 cells to UTP, ATP, ADP, UDP and 2MeSATP. Cells were preincubated in 10 mmol l^{-1} LiCl for 10 min and incubated with agonist and 10 mM LiCl for 15 min. (b) [^{3}H]-inositol (poly)phosphates response of P2Y$_4$ 1321N1 cells to UDP in the absence (control) and presence of hexokinase and glucose. Stock solutions of UDP (5 mM) were incubated for 1 h with hexokinase (50 units ml^{-1}) and glucose (110 mM). P2Y$_4$ 1321N1 cells were preincubated in hexokinase (1 unit ml^{-1}), glucose (22 mM) and LiCl (10 mM); or with LiCl (10 mM) alone (controls) for 10 min. Cells were then incubated in hexokinase-treated UDP and untreated UDP (control) with 10 mM LiCl for 15 min. Each point represents the mean percentage maximal response to ATP from three experiments, each performed in triplicate; vertical lines show s.e.mean.

Discussion

In their original account of the cloning of a novel P2Y receptor expressed in rat aorta cells Chang *et al*. (1995) described how they cloned both P2Y$_2$ and P2Y$_6$ from their cDNA library. The RT–PCR data found in the present study are consistent with this. We describe the presence of mRNA encoding for P2Y$_2$

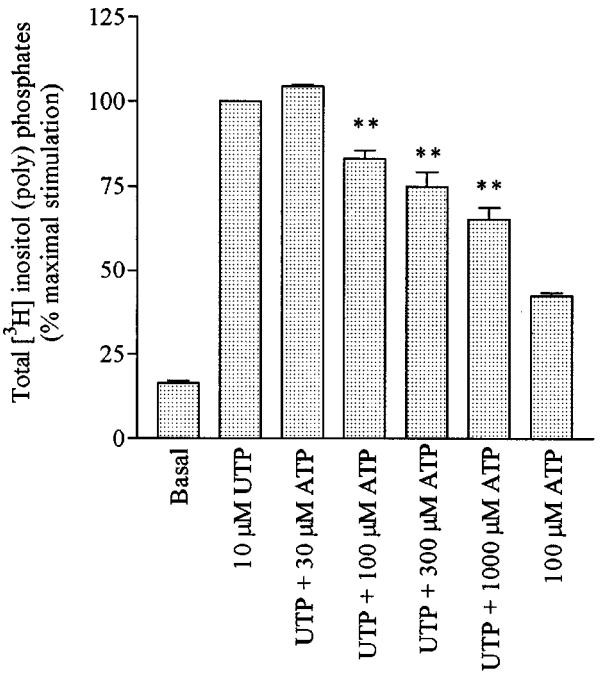

Figure 8 Total [^{3}H]-inositol (poly)phosphate accumulation in $P2Y_4$ transfected 1321N1 cells. Cells were preincubated with 10 mM LiCl and stimulated with UTP (100 μM), ATP (100 μM), or UTP (10 μM) plus ATP (30, 100, 300 and 1000 μM) for 15 min. Each column represents the mean percentage maximal response from three different experiments, each performed in triplicate.

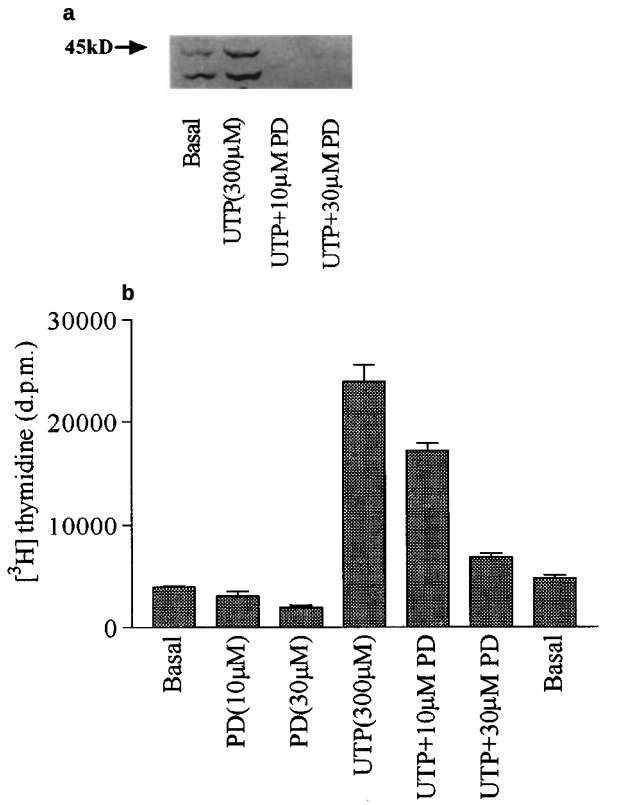

Figure 9 (a) MAPK phosphorylation in SHR-VSM cells. Cells were preincubated in BSS and incubated in BSS, preincubated in BSS and incubated in 300 μM UTP, preincubated in 10 μM PD098059 and incubated in 300 μM UTP, or preincubated in 10 μM PD098059 and incubated in 300 μM UTP. Preincubations were for 20 min and incubations were for 5 min. Data are representative of three experiments. (b) The effect of PD098059 on basal and 300 μM UTP-induced [^{3}H]-thymidine incorporation in SHR-VSMC. Cells were preincubated for 20 min in 10 μM PD098059, 30 μM PD098059, or without antagonist. Incubations with 300 μM UTP or BSS were for 1 h, following which, cells were incubated for a further 19 h in the absence of agonists and antagonists before cell responses were terminated. Each column represents the mean percentage maximal response from three different experiments, each performed in triplicate.

and $P2Y_6$ in these cells. No evidence for transcripts for $P2Y_1$ was seen. However, in agreement with the pharmacology described here, we also determined the expression of mRNA for $P2Y_4$ receptors. This is discussed further below.

Chang *et al.* (1995) characterized the $P2Y_6$ receptor as being a UTP-sensitive pyrimidinoceptor, and then proposed a role for $P2Y_6$ in the PLC and mitogenic response of VSM to nucleotides. The results presented here clearly exclude a major role for $P2Y_6$ receptors in the PLC responses of cultured VSM derived from the aorta of the SHR rat. Nicholas *et al.* (1996) found that UDP was a more potent agonist at $P2Y_6$ receptors than UTP, and Harden *et al.* (1996) concluded that $P2Y_6$ is highly selective for UDP compared to UTP. These studies showed that the responses to the addition of UDP at the cloned and transfected $P2Y_2$ or $P2Y_4$ receptors were lost when hexokinase was used to remove UTP, but that hexokinase had no effect on the $P2Y_6$ response to UDP. In our laboratory we have confirmed the effect of hexokinase on the cloned and transfected receptors, and in parallel experiments found that hexokinase eliminates most of the PLC response to UDP in both transfected $P2Y_4$ receptors and the SHR derived VSM cells. We conclude that UTP, but not UDP, is a potent agonist, and that the receptor responsible is not $P2Y_6$. This is further indicated by the effect of ATP, which Communi *et al.* (1996) found to be ineffectual on transfected human $P2Y_6$ receptors at a concentration of 100 μM, and Nicholas *et al.* (1996) found to be ineffectual on transfected rat $P2Y_6$ receptors at 1 μM. This is in contrast to our observation on both transfected $P2Y_4$ and SHR-derived VSM cells that ATP is maximally effective as a partial agonist at 100 μM. In conclusion the results show that the response we see is not predominantly at $P2Y_6$ receptors.

If the response were due to the simultaneous presence of $P2Y_2$ and $P2Y_6$ receptors, then ATP in the presence of UTP would give the same response as UTP alone. The results presented here show that the presence of ATP reduces the response to UTP, inconsistent with the response being at $P2Y_6$ in addition to $P2Y_2$. This result is also inconsistent with any response at two receptors, one selective for UTP (e.g. $P2Y_4$) and one selective for ATP; this would give an additive effect. However, the results of this experiment are consistent with the presence of a single receptor for both ATP and UTP for which ATP is a partial agonist.

It has previously been shown that ATP is a partial agonist at cloned and transfected $P2Y_4$ receptors (Communi *et al.*, 1995). Using cells transfected with $P2Y_4$ receptors, we showed that the presence of ATP reduces the response to UTP: i.e. it acts as an antagonist. This is inconsistent with the account of an indirect action of ATP in generating a response in $P2Y_4$ transfected 1321N1 cells proposed by Lazarowski *et al.* (1997).

These experiments with the $P2Y_4$ transfectants generated a pattern of agonist responses which closely replicated those seen with the SHR derived VSM cells. Both 1321N1 cells transfected with $P2Y_4$ receptors and the SHR-VSM cells generated the same relative response to UTP and ATP at maximally effective concentrations. However, with the transfected system the EC_{50} for ATP was lower than that for

UTP, while for the SHR-WKY cells they were the same. The EC_{50} for ATP was the same in the two cell types. This is what would be expected if the two cell types had identical receptors but with a receptor reserve only in the case of the transfected system, with UTP as a full agonist but ATP as a partial agonist. The recruitment of a receptor reserve would have no effect on the EC_{50} for a partial agonist, which would have the same EC_{50} in the 2 cell types. This was seen with ATP. However, with a full agonist the presence of a receptor reserve would move the concentration-response curve to the left, and the EC_{50} would thus differ between the cell types. This was seen with UTP.

Taken together these data are consistent with the hypothesis that a single receptor type, the $P2Y_4$ receptor, is responsible for the response in the SHR-derived VSM cells. The studies with antagonists are in agreement with this in that NF023 and PPADS failed to affect the responses to UTP, and that suramin did not alter the EC_{50} for UTP (as seen with 1321N1 cells transfected with $P2Y_4$ receptors by Charlton *et al*., 1995). However, some involvement of $P2Y_2$ receptors in the response of SHR derived VSM cannot be excluded. By contrast, in the WKY-derived cells the maximal responses to ATP and UTP were the same, and the data are consistent with a response predominantly at the $P2Y_2$ receptor. This difference between the SHR and WKY cells may, therefore, reflect a difference in the nature of the two cells with respect to expression of P2Y receptor subtypes. However, it may reflect a differential coupling efficiency between the cell types. If SHR cells have a greater capacity to respond, due to a greater capacity of the signalling process between the receptor and the formation of inositol (poly)phosphates, then a greater efficacy of UTP may become apparent only in the SHR cells. In the WKY cells, ATP may stimulate the cells to their full capacity to respond (via this particular receptor coupling) and thus may elicit a full response, even though its efficacy is lower than that of UTP. Consistent with this interpretation, when WKY derived cells were studied we found that ATP and UTP both gave smaller responses than with SHR derived cells. This explanation is also consistent with an upregulation, at the level of the intracellular signalling cascade, seen with SHR-derived cells when stimulated with angiotensin II (Resink *et al*., 1989; Paquet *et al*., 1990; Osani & Dunn, 1992; Morton *et al*., 1995; Baines *et al*., 1996; Wilkie *et al*., 1997).

We have also shown here that nucleotides stimulate mitogenesis in these cells; the response in WKY cells was small and difficult to study, while the response in SHR derived cells was robust. While we have not characterized the [^{3}H]-thymidine response, the PLC data presented here indicate that the hypothesis that the $P2Y_4$ receptor is involved in the mitogenic response to nucleotides in the SHR derived cells should be further investigated. We have shown here that the nucleotide receptor(s) are coupled to p42 and p44 MAPK. PD 98059 is a selective inhibitor of the pathway upstream of MAPK (Alessi *et al*., 1995) which we have previously shown to have no effect on phospholipase C responses to agonists acting at P2Y receptors (Patel *et al*., 1996). Here we showed that PD 98059 inhibits both the p42 and p44 MAPK response and the [^{3}H]-thymidine response in the SHR derived VSM cells. This outcome provides support for the hypothesis that MAPK activation is a necessary part of the signalling process from the P2Y receptor to the mitogenic response, and the PLC data presented here suggest that the receptor involved may be $P2Y_4$.

The major conclusion of this study concerns the dominant role of $P2Y_4$ receptors in defining the PLC response of the SHR derived cells. The RT–PCR is consistent with this conclusion to the extent that we describe the expression of mRNA encoding for $P2Y_4$ in these cells. This should be seen in the context of essentially undetectable expression of mRNA for $P2Y_1$, but also the presence of mRNA for $P2Y_2$ and $P2Y_6$ receptors. A minor role for $P2Y_2$ receptors cannot be excluded, but the results are inconsistent with a substantial involvement of $P2Y_6$ receptors. It seems that while $P2Y_2$ and $P2Y_6$ receptors are expressed at the messenger level, they are not expressed as functional receptors effectively coupled to PLC.

These observations are of importance in understanding the regulation of contractile and proliferative responses of VSM cells, in particular under those pathological conditions in which smooth muscle proliferation participates. The results may reflect a contribution of $P2Y_4$ receptor signalling via MAPK in hypertensive disease, atherosclerotic plaque formation, and proliferative responses following balloon angioplasty and coronary bypass procedures.

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