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ATP and UTP responses of cultured rat aortic smooth muscle cells revisited: dominance of $P2Y_2$ receptors

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1 It has previously been shown that ATP and UTP stimulate P2Y receptors in vascular smooth muscle cells (VSMCs), but the nature of these receptors, in particular the contribution of $P2Y_2$ and $P2Y_4$ subtypes, has not been firmly established. Here we undertake a further pharmacological analysis of [^{3}H]inositol polyphosphate responses to nucleotides in cultured rat VSMCs.

2 ATP generated a response that was partial compared to UTP, as reported earlier.

3 In the presence of a creatine phosphokinase (CPK) system for regenerating nucleoside triphosphates, the response to ATP was increased, the response to UTP was unchanged, and the difference between UTP and ATP concentration–response curves disappeared. Chromatographic analysis showed that ATP was degraded slightly faster than UTP.

4 The response to UDP was always smaller than that to UTP, but with a shallow slope and a high potency component. In the presence of hexokinase (which prevents the accumulation of ATP/UTP from ADP/UDP), the maximum response to UDP was reduced and the high-potency component of the curve was retained. By contrast, the response to ADP was weaker throughout in the presence of hexokinase.

5 ATPγS was an effective agonist with a similar EC_{50} to UTP, but with a lower maximum. ITP was a weak agonist compared with UTP.

6 Suramin was an effective antagonist of the response to UTP ($pA_2 = 4.48$), but not when ATP was the agonist. However, suramin was an effective antagonist ($pA_2 = 4.45$) when stimulation with ATP was in the presence of the CPK regenerating system.

7 Taken together with the results of others, these findings indicate that the response of cultured rat VSMCs to UTP and to ATP is predominantly at the $P2Y_2$ receptor, and that there is also a response to UDP at the $P2Y_6$ receptor.
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Keywords: P2 receptor; P2Y receptor; $P2Y_2$ receptor; $P2Y_4$ receptor; ATPγS; vascular smooth muscle cells

Abbreviations: ATPγ, adenosine 3′-*O*-(3-thiotriphosphate); CPK, creatine phosphokinase; SHR, spontaneously hypertensive rat; RT–PCR, reverse transcriptase–polymerase chain reaction; VSMCs, vascular smooth muscle cells; WKY, Wistar Kyoto rat

Introduction

Receptor regulation controls two major functions of vascular smooth muscle cells (VSMCs) – the vasomotor contractile response and the proliferative response. These are often considered to be specific to the contractile and synthetic phenotypes, respectively. Contractile phenotypes are mainly studied as *in situ* cells within preparations of vasculature, while synthetic phenotypes, studied as cells in culture, may be taken as models of vascular proliferative disease processes. P2Y receptors, the G protein-coupled receptor family for nucleotides, are known to contribute to the control of both these aspects of VSMC function (e.g., Boarder & Hourani, 1998; McLaren *et al*., 1998; Malmsjo *et al*., 2000; Burnstock, 2002; Chootip *et al*., 2002; Di Virgilio & Solini, 2002; Vial & Evans, 2002).

Studies of the synthetic phenotype using cultured rat aorta VSMC have shown that ATP, UTP and UDP activated various proliferative signalling pathways downstream of P2Y receptor activation (Malam-Souley *et al*., 1993; Erlinge *et al*., 1993; Miyagi *et al*., 1996; Erlinge, 1998; Harper *et al*., 1998; Hou *et al*., 2002; Pillois *et al*., 2002; Seye *et al*., 2002). Erlinge (1998) undertook a quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) study comparing the expression of transcripts for P2Y receptors in contractile and synthetic forms of rat aorta cells. They showed that $P2Y_2$ and $P2Y_6$ had similar expression levels in the contractile forms, with $P2Y_1$ and $P2Y_4$ present at lower levels. In the synthetic phenotype, $P2Y_1$ and $P2Y_2$ were upregulated and became the most abundant, with $P2Y_4$ and $P2Y_6$ also present. Direct evidence of a role for $P2Y_6$ receptors was provided by Hou *et al*., (2002) Using cultured rat cells, they provided evidence that the $P2Y_6$ receptor is functional and responsible for the mitogenic response to UDP. This followed an earlier report of ours in which we showed that the response to ATP was partial

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compared to UTP when measuring phosphoinositidase C activity in cultured rat aortic VSMCs (Harper *et al*., 1998). This was interpreted as evidence for a response at $P2Y_4$ receptors in light of the currently available evidence that ATP was a partial agonist at these receptors (Communi *et al*., 1995). However, around this time, it emerged that there are species differences between rat (r) and human (h) $P2Y_4$ receptors – ATP displays low efficacy at $hP2Y_4$ receptors, but is a full agonist at $rP2Y_4$ receptors (Bogdanov *et al*., 1998; Webb *et al*., 1998; Kennedy *et al*., 2000). In this respect, the responses at the $rP2Y_4$ receptor mirror those at the $rP2Y_2$ receptor. The previous conclusion that the responses to ATP and UTP of cultured rat aortic VSMC can be explained by the presence of $P2Y_4$ receptors has therefore been re-evaluated. Distinguishing between the involvement of $rP2Y_2$ and $rP2Y_4$ receptors is difficult. In this paper, we have characterised the responses to purified nucleotides, including ATPγS and ITP, as well as the contribution of agonist interconversion to the partial response to ATP and its sensitivity to suramin. Taking these evidences together, we conclude that the response to ATP and UTP of cultured rat aortic VSMC is most likely to be dominated by the $P2Y_2$ receptor.

Methods

Rat aortic VSMCs from both spontaneously hypertensive (SHR) and normotensive (WKY) animals were prepared as described in Davies *et al*. (1991). Briefly, the arterial blood pressures of 12-week-old SHR and WKY rats were determined using tail cuff measurements, following which the rats were decapitated. Thoracic aorta was denuded of adventitia, cut into 1 mm cubes and digested. Liberated cells were cultured in Dulbecco's modified Eagle's medium with 10% foetal calf serum, penicillin (50 IU ml^{-1}), streptomycin (50 μg ml^{-1}) and L-glutamine (2 mM). Clonal cell lines were established, and those showing smooth muscle morphology combined to generate cell cultures used in this study. These cultures were 100% positive for smooth muscle actin immunofluorescence.

1321N1 cells stably expressing $hP2Y_2$ and $hP2Y_4$ receptors were a kind gift of Dr T.K. Harden, University of North Carolina. They were cultured in Dulbecco's modified Eagle's medium with 10% foetal calf serum, penicillin (50 IU ml^{-1}), streptomycin (50 μg ml^{-1}) and L-glutamine (2 mM).

For the total [^{3}H]-inositol (poly)phosphate measurements, cells were cultured to 80% confluence in 24-well multiwell plates. The medium was then replaced with 500 μl of serum-free M199 medium supplemented with 50 IU ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 2 mM L-glutamine and 1 μCi ml^{-1} (0.037 MBq ml^{-1}) D-*myo*-[2-3 H]inositol. After 24 h, cells were incubated with 10 mM LiCl for 10 min – where appropriate suramin was included with the lithium. After 20 min stimulation with agonists in the continuing presence of lithium, the reaction was stopped with trichloroacetic acid, the solution was neutralised with freon : octylamine extraction, and addition of $NaHCO_3$ and [^{3}H]inositol phosphates separated on small Dowex-1 columns.

HPLC was used to purify all agonists prior to use, to monitor the effects of hexokinase, creatine phosphokinase (CPK) and apyrase, and to monitor the nucleotide breakdown by the cultured cells. An Alltech SAX column was used with a Waters HPLC system (gradient between 0.05 M KH_2PO_4 and 1 M KH_2PO_4), with data collection and peak integration by Millemium 32 software. Stock solutions of ADP and UDP were treated with 50 U ml^{-1} of hexokinase and 110 mM glucose for 1 h at 37°C immediately before use. In addition, cells were incubated with 1 U ml^{-1} hexokinase and 22 mM glucose for 1 h prior to stimulation (including the 10 min with lithium) and during the 20 min stimulation. For the CPK-regenerating system, agonists and cells were preincubated for 10 min, and during stimulation, with 2 U ml^{-1} CPK and 1 mM phosphocreatine. ATPγS was preincubated immediately before use with 20 U ml^{-1} apyrase (Sigma) at 30° for 1 h, following which the apyrase was denatured by heating to 100°C for 5 min. HPLC confirmed that ADP which contaminated some stocks of ATPγS was removed by apyrase treatment.

For breakdown studies, confluent SHR-derived cells in 24-well multiwells were stimulated with 300 μM ATP or UTP in a final volume of 300 μl at 37°C for the times indicated. Supernatants were transferred to tubes on ice containing 50 μl 3.5 M trichloroacetic acid and 100 μl aliquots were analysed by HPLC. Integration of the chromatogram generated an area under the ATP or UTP peak, which was expressed as a percentage of the area when the incubation time was close to zero (agonists added to cells then immediately removed). Data presented are mean ± s.e.m. pooled from three separate experiments.

For the estimation of pA_2 values for suramin, the method of Furchgott was used: $pA_2 = \log_{10}(r-1) + p[A]$, where r is the dose ratio of EC_{50} value in the presence of antagonist compared to its absence, and p[A] is the $-\log_{10}$ of the molar agonist concentration. For statistical analysis, the EC_{50} values were converted to pEC_{50} ($-\log_{10} EC_{50}$). Statistical analysis and curve fitting were carried out by the Graph Pad Prism.

Results

Use of SHR-derived VSMC

We have previously reported that the VSMC derived from SHRs generated larger responses to nucleotides than those from normotensive WKY control rats. In Figure 1, we show this to be true of the present study when measuring [^{3}H]InsP_x levels in response to UTP. The maximal response to UTP was 212.7 ± 39.5 dpm μg^{-1} protein for SHR-derived cells and 61.5 ± 14.7 dpm μg^{-1} protein for WKY-derived cells ($P<0.001$, two-way ANOVA followed by Bonferonni's post-test). The EC_{50} for UTP was the same for either cell preparation ($pEC_{50} = 5.00 \pm 0.04$ and 4.99 ± 0.04 for SHR and WKY cells, respectively). In Figure 2, we show concentration–response curves for a variety of nucleotide agonists for the SHR-derived cells. We have performed identical analyses of responses to WKY cells, and conclude that there is no significant difference in the relative responses to different agonists between WKY- and SHR-derived cells. For the remainder of the work reported here, we used SHR-derived cells, since the larger response of these cells made them easier to study.

Response to UTP and ATP

Figure 2 shows that ATP produced a response which was partial compared to that of UTP, as previously reported

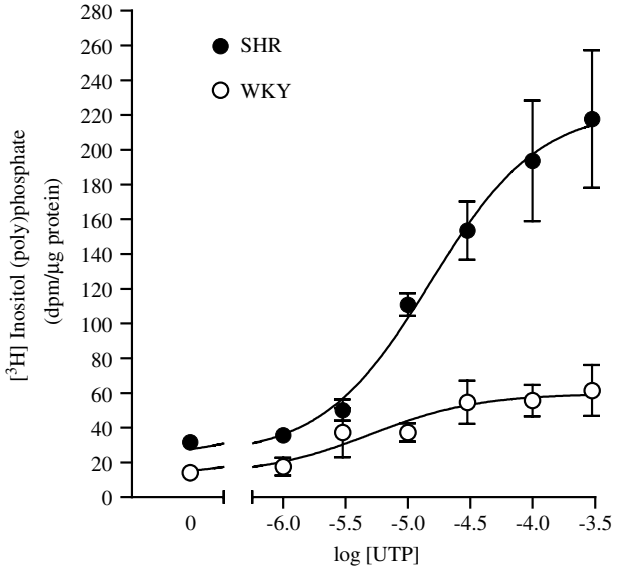

Figure 1 Concentration–response curves of the total [^{3}H]inositol polyphosphate accumulation, as an indication of PLC activation, in SHR- and WKY-derived VSMCs, in response to increasing concentration of UTP. Cells were preincubated with 10 mM LiCl for 10 min and incubated with a concentration range of 1–300 M UTP plus 10 mM LiCl for 20 min. The dpm of [^{3}H]inositol (poly)phosphates formed are expressed per μg of protein from each cell culture. Each point represents the mean of three experiments from separate cell cultures, performed in triplicate.

(Harper *et al*., 1998). To investigate whether this is a result of differential breakdown in the extracellular compartment, we have constructed concentration–response curves for UTP and ATP in the presence of the CPK-regenerating system, which effectively prevents accumulation of nucleoside diphosphates and the loss of triphosphates. CPK alone had no effect (data not shown). Figure 3a shows that responses to UTP were not significantly affected by CPK. Contrasting with this, the responses to ATP (Figure 3b) were enhanced by CPK throughout the concentration range ($P<0.0001$ by two-way ANOVA). In the presence of CPK, the concentration–response curve to ATP was not significantly different from that of UTP; the maximal response to ATP with CPK was $96.0\pm9.4\%$ of that to UTP. The pEC_{50} for ATP with CPK was 5.23 ± 0.14, compared to a pEC_{50} of 5.44 ± 0.07 for UTP.

Breakdown of ATP and UTP

The selective effect of CPK, enhancing the ATP response while leaving the UTP response unaffected, suggested that differential breakdown may play a role in the relative responses to ATP and UTP. We used HPLC analysis to investigate the loss of ATP and UTP on incubation with confluent SHR-derived cultures. During these investigations, we saw no accumulation of di- or monophosphates, presumably because they were further metabolised as they were formed. Figure 4 shows that there was a slow rate of decline of both ATP and UTP concentrations over 30 min, and that the rate of loss of ATP was faster than that of UTP ($P<0.001$ by two-way ANOVA). At 20 min, the concentration of UTP had fallen by $21.0\pm2.1\%$, compared to $39.5\pm1.8\%$ for ATP. Analysis of the curves for the concentrations of ATP and UTP showed

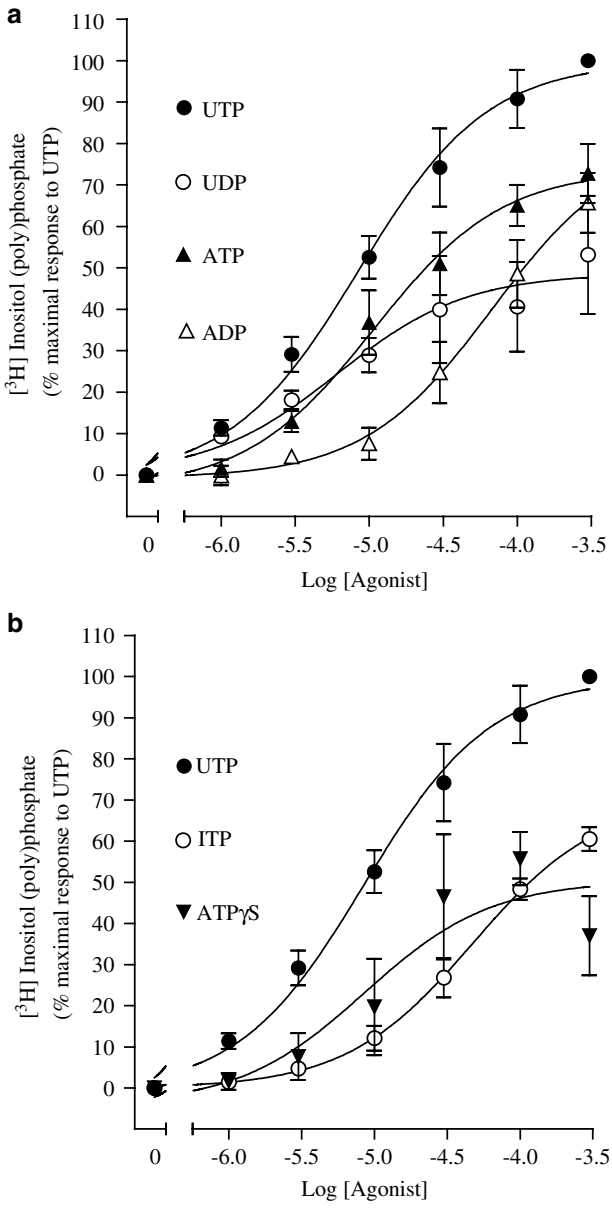

Figure 2 Concentration–response curve of total [^{3}H]inositol polyphosphate accumulation in rat VSMC to (a) purified UTP, UDP, ATP, ADP and (b) purified UTP, ITP and ATPγS. The data forming the curve for UTP are the same for both (a) and (b). Cells were preincubated with 10mM LiCl for 10 min and incubated with agonist plus 10 mM LiCl for 20 min. Each point represents the mean % maximal response to UTP from four experiments, each performed in triplicate.

that, integrated over 20 min, ATP in the medium was 89.5% of the UTP concentration.

Response to UDP and ADP

As with UTP, the response to UDP was greater in SHR- than in WKY-derived cells. However, when expressed as a percentage of the maximal response to UTP, the maximal responses to UDP were the same for the two cell types (48.6 ± 6.2 and $45.5\pm2.5\%$, respectively). Further analysis was undertaken on the SHR-derived cells. The concentration–

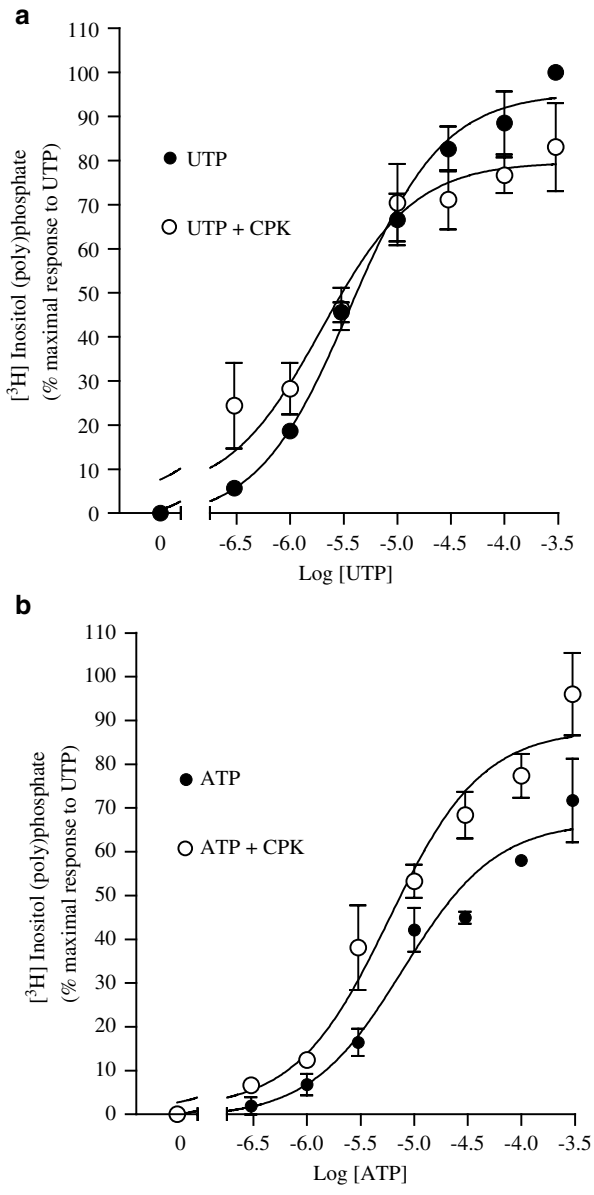

Figure 3 Concentration–response curve of total [^{3}H]inositol polyphosphate accumulation in rat VSMC, stimulated with (a) UTP, UTP + CPK and (b) ATP and ATP + CPK. Cells were preincubated with 10 mM LiCl for 10 min and stimulated with agonist plus, where appropriate, 2 U ml^{-1} CPK, 1 mM phosphocreatine and 10 mM LiCl for 20 min. Each point represents the mean % maximal response from three experiments, each performed in triplicate.

response curve to UDP had a low slope, showing evidence of both low- and high-potency components (Figures 2a and 5a). The curve failed to form a plateau at the highest concentration used, but taking the response to this concentration as the maximal response yields an apparent EC_{50} of 7.2 μM (pEC_{50} 5.14 $\pm$ 0.16). The presence of hexokinase reduced the maximal response to UDP (to 28.6 $\pm$ 0.6% of the response to UTP) and at the same time shifted the EC_{50} to the left ($pEC_{50} = 6.39 \pm 0.13$, $EC_{50} = 410$ nM) with loss of the low-potency component (Figure 5a).

The effect of hexokinase on the response to UDP was in sharp contrast to the effect when stimulation was with ADP. In the absence of hexokinase, ADP gave a response which was

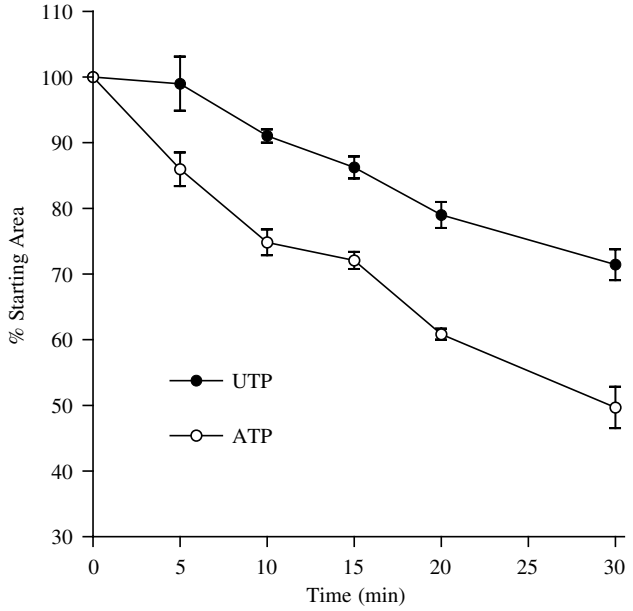

Figure 4 Breakdown of ATP and UTP by SHR-derived cells. Incubation of 300 μM of each nucleotide was carried out for the times incubated, followed by HPLC analysis. Integration generated an area under the ATP and UTP peak in arbitrary units, which was then expressed as a percentage of the area in the absence of incubation (% starting area) for each time point. Data were pooled across three separate experiments and expressed as mean $\pm$ s.e.m.

substantially lower than that for UTP, and which failed to reliably show signs of reaching a plateau (Figure 5b), precluding estimation of an EC_{50}. In the presence of hexokinase, the response at each concentration of ADP was reduced, with the lower concentrations failing to elicit a significant response.

Responses to ATPγS and ITP

Using transfected 1321N1 cells expressing $hP2Y_2$ and $hP2Y_4$ receptors, we have established that ATPγS was a full agonist at $hP2Y_2$ receptors (Figure 6a). However, ATPγS was not an effective agonist (Figure 6b) or antagonist (Figure 6c) at $hP2Y_4$ receptors. Figure 6b compares the responses to ATPγS, ATP and UTP in 1321N1 cells expressing $hP2Y_4$ receptors. Only a partial concentration–response curve to UTP is shown in Figure 6b, which shows that ATP acts as a weak apparent agonist, while ATPγS has no effect at all. Full concentration–response curves for UTP are presented in Figure 6c, where it is apparent that inclusion of either 10 or 100 μM ATPγS had no effect on the response to UTP. Species differences, particularly for $P2Y_4$ receptors, indicate that these findings for human P2Y receptors must be used with caution in the interpretation of results from experiments on rat-derived cells (see Discussion). However, it is reported by Bogdanov *et al*. (1998) that $rP2Y_4$ receptors are not activated by ATPγS. Here we report (Figure 2b) that ATPγS was an effective agonist at rat VSMCs. ATPγS had a similar potency to UTP, with an EC_{50} of 11.9 μM ($pEC_{50} = 4.92 \pm 0.04$), although the maximum response to ATPγS was less than that to UTP (50.4 $\pm$ 7.4% for SHR-derived cells and 61.1 $\pm$ 1.8% for WKY-derived cells).

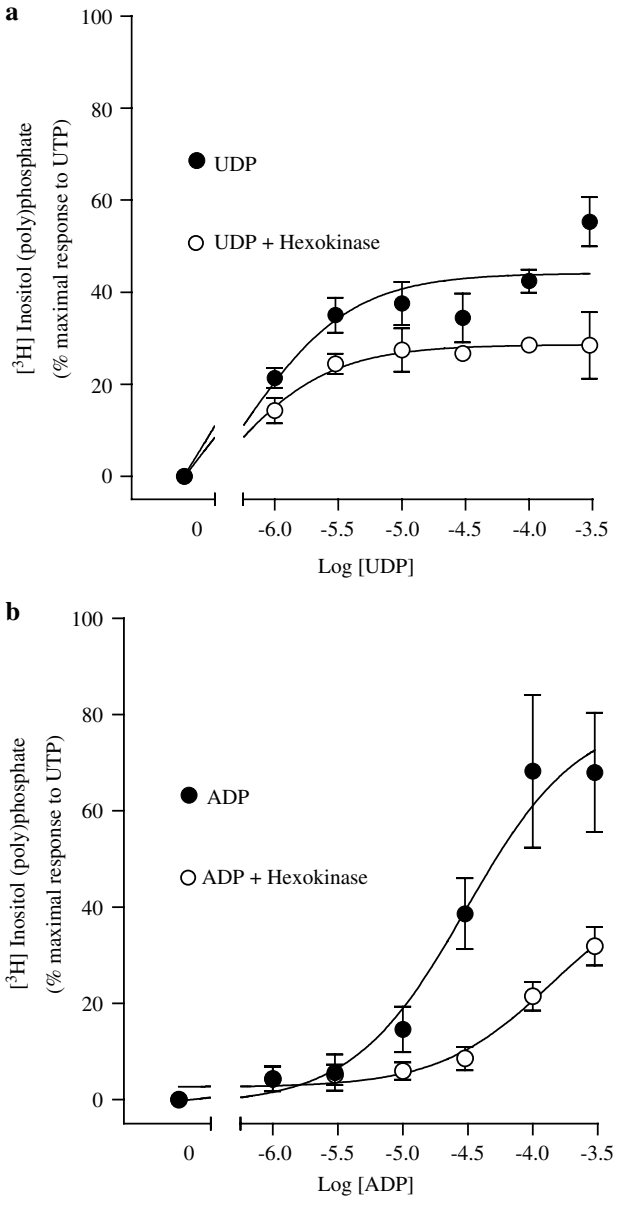

Figure 5 Concentration–response curve of total [^{3}H]inositol polyphosphate accumulation in rat VSMC, (a) to purified UDP and purified UDP + hexokinase, and (b) to purified ADP and purified ADP + hexokinase. Cells were preincubated with 50 U ml^{-1} hexokinase, 110 mM glucose and 10 mM LiCl for 10 min, and incubated with agonist plus 1 U ml^{-1} hexokinase, 22 mM glucose and 10 mM LiCl for 20 min. Each point represents the mean % maximal response to UTP (not shown) from three experiments, each performed in triplicate.

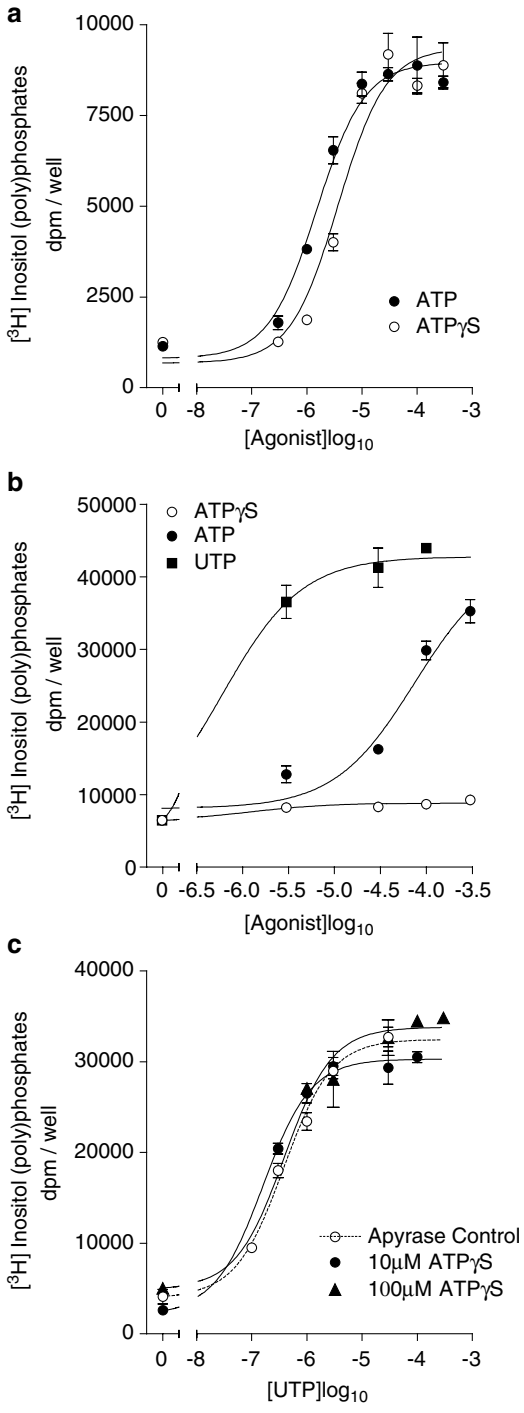

Figure 6 Effect of ATPγS on human P2Y$_2$ and P2Y$_4$ receptors. 1321N1 cells expressing hP2Y$_2$ (a) and hP2Y$_4$ (b) receptors were stimulated with agonists as shown, and the accumulation of [^{3}H]inositol (poly)phosphates was recorded. (a) Concentration–response curves to ATP and ATPγS in P2Y$_2$-expressing cells. (b) Concentration–response curves to ATP, UTP and ATPγS in P2Y$_4$-expressing cells. (c) Concentration–response curves to UTP in P2Y$_4$-expressing cells in the presence of apyrase-treated ATPγS at either 10 or 100 μM, compared to apyrase controls. In each case, the apyrase was inactivated by heating to 100°C prior to contact with UTP, as described in Methods. Data are mean ± s.e.m. ($n = 3$) from a single representative experiment.

P2Y$_2$ and P2Y$_4$ receptors have been thought to differ in their responses to ITP (see Discussion). Figure 2b shows that ITP acts as an agonist of weak potency at rat VSMC, failing to approach a plateau at the maximal concentration used. At 300 μM, the response to ITP was 74.6 ± 5.0% of the response to 300 μM UTP. Extrapolation of this curve gave an apparent EC_{50} value of 59.5 μM ($pEC_{50} = 4.23 \pm 0.05$). The ratio of EC_{50} between UTP and ITP can then be calculated as 10.1/59.5 = 0.169.

Effects of suramin

Previously, we reported that the response to UTP was attenuated by suramin. Here we show that the response to ATP was unaffected by this antagonist (Figure 7a). This observation was unexpected. In view of the demonstration (Figure 3b) that the response to ATP was enhanced by CPK, we tested the effect of suramin on ATP stimulation in the presence of CPK. We found that the CPK-enhanced response to ATP was antagonised by suramin (Figure 7b). In parallel experiments, we constructed concentration–response curves to UTP and ITP in the presence and absence of suramin (not shown). We found that the pA_2 values for suramin were essentially the same whether the agonist was ATP plus CPK, UTP or ITP (pA_2 = 4.45, 4.48 or 4.75, respectively).

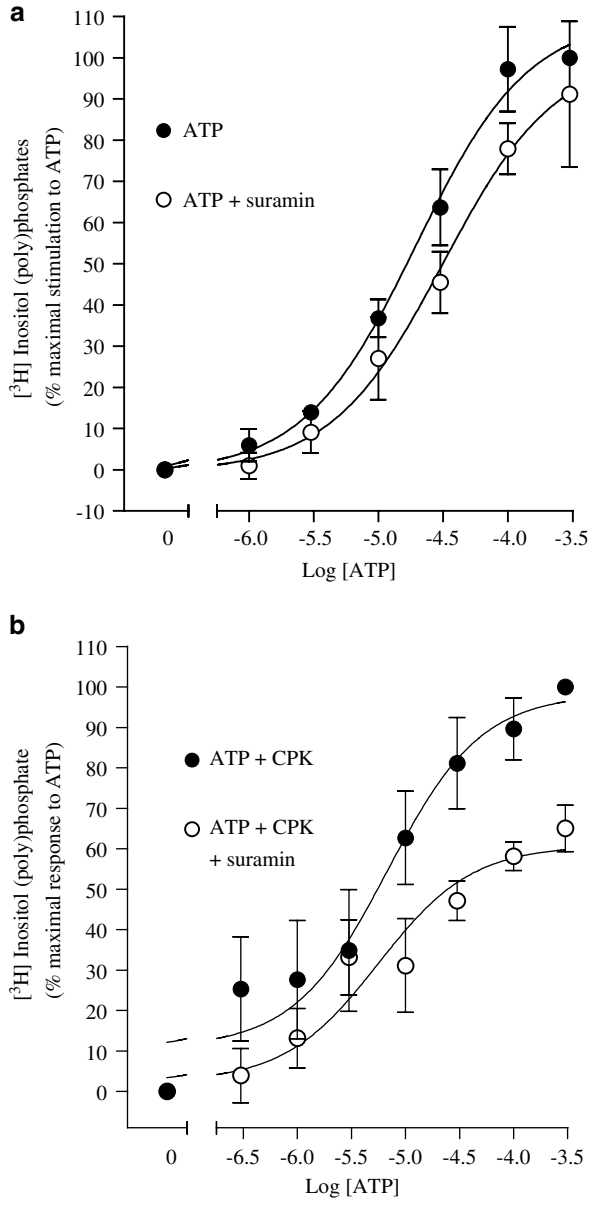

Figure 7 Concentration–response curve of total [^{3}H]inositol polyphosphate accumulation in rat VSMC in response to (a) ATP, and ATP plus 300 μM suramin; (b) ATP in the presence of CPK, and ATP in the presence of CPK plus 300 μM suramin. Each point represents the mean % maximal response as indicated from three/four experiments, each performed in triplicate.

Discussion

In this paper, we have used SHR-derived aortic cells in culture as a model of the synthetic phenotype of rat VSMCs, in order to clarify the pharmacology of responses to nucleotides. Our analysis, comparing the SHR-derived cells with those from normotensive rats, leads to the conclusion that the P2Y pharmacology is the same for the two cell types. However, the responses of SHR-derived cells were larger than those of normotensive WKY-derived cells, consistent with earlier reports (e.g. Resink *et al*., 1989; Paquet *et al*., 1990; Osani & Dunn, 1992; Morton *et al*., 1995; Baines *et al*., 1996; Harper *et al*., 1998).

The concentration–response curves presented here confirm the earlier observation that UTP generates a larger maximum response than ATP (Harper *et al*., 1998). This was interpreted as evidence for involvement of $P2Y_4$ receptors, since studies on human $P2Y_4$ had shown ATP to be less efficacious than UTP (Communi *et al*., 1995). The subsequent demonstration that rat $P2Y_4$ receptors responded with equal efficacy to ATP and UTP (Bogdanov *et al*., 1998; Webb *et al*., 1998; Kennedy *et al*., 2000) led us to seek an alternative explanation. The key observation came from using chromatographically purified ATP and UTP together with a CPK-regenerating system. This converts ADP or UDP formed in the extracellular compartment during the incubation back into ATP and UTP, simultaneously preventing loss of triphosphates and formation of other agonists through breakdown. Under these conditions, the response to ATP was increased, and concentration–response curves to the two agonists became essentially the same. This suggests that the breakdown of ATP in the pericellular space is greater than that of UTP. HPLC analysis clearly established that ATP is broken down at the cell surface faster than UTP. The difference in bulk phase concentration (integrated over 20 min) of only 10.5% is apparently insufficient to account for the difference in response to ATP and UTP. However, there will be a greater difference in concentration between the two agonists at the cell surface, where the receptors and the ATPase activity are located, than recorded here in the bulk phase.

These findings indicate that the responses to ATP and UTP in rat VSMC could be mediated by either $P2Y_2$ or $P2Y_4$ receptors. We have shown here that ATPγS is not an agonist at the transfected $hP2Y_4$ receptor. This is of interest in that it is consistent with the conclusion of Kennedy *et al*. (2000) that the weak apparent agonist action of ATP at $hP2Y_4$ receptors is dependent on its activity as a phosphate donor in the extracellular formation of UTP. However, Kennedy *et al*. (2000) also report that ATP acts as an antagonist at $hP2Y_4$ receptors. Here we show that ATPγS differs from ATP in its lack of antagonist action, showing that ATPγS acts in a fundamentally different way from ATP in that it does not occupy the agonist-binding site of the $hP2Y_4$ receptor. It is conceivable, therefore, that ATPγS may show a similar difference to ATP at the $rP2Y_4$ receptor. Indeed, Bogdanov *et al*. (1998) showed that, in oocytes expressing $rP2Y_4$ receptors, ATPγS was not an effective agonist. By contrast, with cloned murine or human $P2Y_2$ receptors, it is reported

that ATPγS is a full agonist with equal potency to ATP or UTP (this report), a full agonist with lower potency than ATP or UTP (Lazarowski *et al*., 1995), or an agonist of lower potency and producing a maximal response a little lower than that of ATP or UTP (Erb *et al*., 1993; Lustig *et al*., 1993). This variation is most likely due to ATPγS acting with lower efficacy than ATP at $P2Y_2$ receptors, combined with differences in receptor reserves for the expression systems used and responses measured. In the present study on native P2Y receptors, we see a maximal response to ATPγS, which is 50–60% of that of UTP. On the basis of the work discussed above, we interpret this as indicating a response at $P2Y_2$ receptors with a low receptor reserve in the synthetic phenotype of the rat VSMC.

A further attempt to characterise the response was made with ITP. Our results show that rat VSMCs exhibit an agonist potency order of UTP > ITP (EC_{50} ratio of UTP/ITP of less than one). Fillippov *et al*. (1997) presented results that give a UTP/ITP ratio of less than one (0.166) for $rP2Y_2$, and Bogdanov *et al*. (1998) reported data yielding a UTP/ITP ratio of greater than one (1.857) for $rP2Y_4$. On this basis, our data are consistent with a response at $P2Y_2$ receptors, although it should be recognised that EC_{50} ratios are not independent of variations in the receptor reserve.

The observation here that suramin does not inhibit the response to ATP was unexpected in view of our earlier report (Harper *et al*., 1998; repeated in the present study – data not shown) that suramin acts as an effective antagonist of the response to UTP. Hourani & Chown (1987) and Chen *et al*. (1996) have shown that suramin is an ectonucleotidase inhibitor; so the lack of effect on the ATP response could be due to two countervailing influences – an antagonist influence at the receptor and a response-enhancing influence due to ectonucleotidase inhibition. To test this, we constructed concentration–response curves to ATP with the CPK-regenerating system, and found that under these conditions suramin acted as an antagonist, with a similar pA_2 value to UTP. Interestingly, the pA_2 values we report for suramin antagonism of responses to ATP (with CPK), UTP and ITP were the same as those we have reported for $hP2Y_2$ ($pA_2 = 4.3$) in a paper which shows that suramin is not an effective antagonist at $hP2Y_4$ (Charlton *et al*., 1996). These results provide strong support for the view that ATP, UTP and ITP all act on $P2Y_2$ receptors in rat VSMCs.

Our observations also demonstrate a substantial contribution by UTP to the UDP response, through extracellular conversion and/or contamination of the nucleotide stock. However, when generation of UTP was eliminated, UDP remained a potent agonist but with a smaller maximal response than UTP or ATP. This response is likely to be mediated by $P2Y_6$ receptors, as proposed by Hou *et al*., (2002).

Taken together, the results presented here and the work of others provide a cogent argument that the response of the synthetic phenotype of rat aortic smooth muscle cells to nucleotides is mainly through $P2Y_2$ receptors, with a smaller contribution from $P2Y_6$ receptors. This is consistent with the outcome predicted by quantitative RT–PCR data of Erlinge *et al*. (1999) and Pillois *et al*. (2002), showing that the most abundant transcripts expressed by the synthetic phenotype of rat VSMC are for $P2Y_2$ receptors. Interestingly, it is also consistent with an *in vivo* study of Seye *et al*. (2002), who showed that injury-induced intimal proliferation in rabbit carotid arteries is associated with $P2Y_2$ upregulation in VSMCs.

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