British Journal of Pharmacology (1996) 118, 704-710

PPADS and suramin as antagonists at cloned P_{2Y}- and P_{2U}-purinoceptors

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1 The effect of suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on the stimulation of phospholipase C in 1321N1 cells transfected with the human P_{2U}-purinoceptor (h-P_{2U}-1321N1 cells) or with the turkey P_{2Y}-purinoceptor (t-P_{2Y}-1321N1 cells) was investigated. 2-Methylthioadenosine triphosphate (2MeSATP) was used as the agonist at t-P_{2Y}-1321N1 cells and uridine triphosphate (UTP) at h-P_{2U}-1321N1 cells.

2 Suramin caused a parallel shift to the right of the concentration-response curves for 2MeSATP in the t-P_{2Y}-1321N1 cells, yielding a Schild plot with a slope of 1.16 ± 0.08 and a pA_2 value of 5.77 ± 0.11.

3 Suramin also caused a shift to the right of concentration-response curves for UTP in the h-P_{2U}-1321N1 cells, and on Schild plots gave a slope different from unity (1.57 ± 0.19) and an apparent pA_2 value of 4.32 ± 0.13. Suramin was therefore a less potent antagonist at the P_{2U}-purinoceptor than the P_{2Y}-purinoceptor.

4 In the presence of the ectonucleotidase inhibitor, ARL 67156 (6-N,N-diethyl-β,γ-dibromomethylene-D-ATP) there was no significant difference in the EC_{50} or shapes of curves with either cell type, and no difference in pA_2 values for suramin.

5 PPADS caused an increase in the EC_{50} for 2MeSATP in the t-P_{2Y}-1321N1 cells. The Schild plot had a slope different from unity (0.55 ± 0.15) and an X-intercept corresponding to an apparent pA_2 of 5.98 ± 0.65.

6 PPADS up to 30 μM had no effect on the concentration-response curve for UTP with the h-P_{2U}-1321N1 cells.

7 In conclusion, suramin and PPADS show clear differences in their action at the 2 receptor types, in each case being substantially more effective as an antagonist at the P_{2Y}-purinoceptor than at the P_{2U}-purinoceptor. Ectonucleotidase breakdown had little influence on the nature of the responses at the two receptor types, or in their differential sensitivity to suramin.

Keywords: P_{2Y}-purinoceptors; P_{2U}-purinoceptors; ectonucleotidases; suramin; PPADS

Introduction

ATP and ADP play a widespread role in cell signalling, acting at cell surface receptors called P_2-purinoceptors and influencing a diverse array of bodily functions (Dubyak & El Moatassim, 1993). Classification based on rank order of agonist potency generated an initial subdivision into P_{2X} and P_{2Y}-purinoceptors (Burnstock & Kennedy, 1985), which are now known to be ion channel and 7-transmembrane G protein-coupled receptors respectively. Subsequent studies have revealed further types of G protein-coupled P_2-purinoceptors, such as the P_{2U}-purinoceptor (O'Connor *et al.*, 1990). In a recent revision of nomenclature, P_{2X}-, P_{2Y}- and P_{2U}-purinoceptors have been designated P2X, $P2Y_1$ and $P2Y_2$ respectively (Fredholm *et al.*, 1994). Recently successful cloning strategies have had a major impact on this area of pharmacology, with the cloning of 3 subtypes of P_{2X}-purinoceptors (Brake *et al.*, 1994; Valera *et al.*, 1994; Chen *et al.*, 1995; Lewis *et al.*, 1995) and cloning of G protein-coupled P_{2Y}- and P_{2U}-purinoceptors from several species (e.g. Webb *et al.*, 1993; Lustig *et al.*, 1993; Filtz *et al.*, 1994; Parr *et al.*, 1994; Tokuyama *et al.*, 1995; Henderson *et al.*, 1995) as well as a pyrimidinoceptor (Communi *et al.*, 1995; Nguyen *et al.*, 1995). The integration of this molecular information into an understanding of the cellular and tissue functions of these receptors is in its early stages (Boarder *et al.*, 1995). A major restraint on this process is the lack of highly selective antagonists, despite some important recent progress (e.g. the development by Humphries *et al.*, 1994, of FPL 66096, a potent and selective P_{2T}-purinoceptor antagonist). Suramin and pyridoxalphosphate-6-azophenyl-2',4'-disulphonoic acid (PPADS), are effective antagonists at P_2-purinoceptors. However, these drugs show limited selectivity for different classes of P_2-purinoceptor. The degree of selectivity which they do exert is poorly understood, perhaps largely due to the use of systems with mixed populations of P_2-purinoceptors, and where agonist breakdown by ectonucleotidases has an uncertain effect on the results. For example, while suramin is widely used as a non-selective competitive P_2-purinoceptor antagonist (e.g. Dunn & Blakely, 1988; Leff *et al.*, 1990; Hoyle *et al.*, 1990) we have shown, in bovine aortic endothelial cells, that it is more effective at P_{2Y}- than at P_{2U}-purinoceptors (Wilkinson *et al.*, 1993; 1994). We and others (Murrin & Boarder, 1992; Dainty *et al.*, 1994; Wilkinson & Boarder, unpublished) have also provided evidence for suramin-sensitive and suramin-insensitive P_{2U}-purinoceptors. However, suramin is known to be an ectonucleotidase inhibitor (Hourani & Chown, 1989), and the role this may play in this apparent selectivity is unclear. With PPADS (only a weak ectonucleotidase inhibitor: Windscheif *et al.*, 1995), it is well established that it is an effective P_{2X} antagonist (e.g. Lambrech *et al.*, 1992; Zighanshin *et al.*, 1994; Trezise *et al.*, 1994). PPADS has also been reported as an

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effective antagonist at turkey erythrocyte, guinea-pig taenia coli and rat duodenum P_{2Y}-purinoceptors, but relatively ineffective at P_{2Y}-purinoceptors of the rat mesenteric arterial bed and rat C6 glioma cells (Windscheif *et al.*, 1994; Boyer *et al.*, 1994), leading to the suggestion of multiple subtypes of P_{2Y}-purinoceptors (Boyer *et al.*, 1994; Windscheif *et al.*, 1995). We have reported that PPADS is an effective antagonist at bovine endothelial P_{2Y}-, but not P_{2U}-purinoceptors (Brown *et al.*, 1995), revealing an apparent further selectivity of this agent. Ho *et al.* (1995) recently found that PPADS can act as an antagonist at astrocyte P_{2U}-purinoceptors, but that it is considerably less potent than at astrocyte P_{2Y}-purinoceptors.

We now have the opportunity to study the action of antagonists at cloned receptors, and thus to avoid the uncertainty of native systems with respect to the nature of the receptors and multiple receptors in the same preparation. In this paper we have used cloned human P_{2U}- and turkey P_{2Y}-purinoceptors stably transfected into a common host cell line, the human astrocytoma 1321N1 cells, as described previously (Parr *et al.*, 1994; Filtz *et al.*, 1994). The influence of ectonucleotidases on the outcome of the experiments has been directly addressed by the use of the ectonucleotidase inhibitor, ARL 67156 (6-N,N-diethyl-β,γ-dibromomethylene-D-ATP) (Crack *et al.*, 1995).

Methods

Lines of 1321N1 cells (with no native responses to P_2-purinoceptor agonists) stably transfected with human P_{2U}-purinoceptors (h-P_{2U}-1321N1 cells) or with turkey P_{2Y}-purinoceptors (t-P_{2Y}-1321N1 cells) have been described previously (Parr *et al.*, 1994; Filtz *et al.*, 1994). These were cultured in high (4 mg ml^{-1}) glucose DMEM with 10% foetal calf serum, 25 iu ml^{-1} penicillin, 25 μg ml^{-1} streptomycin, 10 mg ml^{-1} gentamycin, at 37°C in 5% CO_2. Cells at confluence in 24 well multiwells were labelled overnight with *myo*-[2-^{3}H]-inositol (1 μCi ml^{-1}, 0.5 ml per well) in medium M199 with glutamine, penicillin and streptomycin at the above concentrations. In preliminary experiments we found that changing the medium of the transfected cells resulted in a substantial stimulation of phospholipase C responses, perhaps due to release of endogenous ATP (Lazarowski *et al.*, 1995). We used two procedures intended to avoid the consequences of this. In the first procedure used, the labelling medium was aspirated and replaced with 300 μl BSS, $\pm$ARL 67156 as appropriate. After 30 min 50 μl of LiCl at 7 fold final concentration (70 mM) was added, with antagonists as indicated. After a further 10 min the agonist was added in 50 μl BSS at 8 fold final concentration. After a subsequent 15 min incubation, 100 μl of 2.5 M trichloroacetic acid was added, and the plates left on ice for 1 h prior to separation of total [^{3}H]-inositol (poly)phosphates on small Dowex-1 (Cl^-) columns. In later studies we used a modified procedure which gave lower basal values. With this protocol the medium was not changed following labelling overnight. To the 0.5 ml of labelling medium was added 50 μl of 11 fold final concentration of LiCl (110 mM) in balanced salt solution (BSS, composition, 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, pH 7.4). Antagonists were added, where appropriate, at 11 fold final concentration with the LiCl. After 10 min incubation at 37°C the agonist was added in 50 μl BSS at 12 fold final concentration. After further incubation for 15 min the medium was aspirated and 0.5 ml of ice cold trichloroacetic acid was immediately added. After leaving on ice for 1 h the trichloroacetic acid was extracted with 3 ether washes, and total [^{3}H]-inositol (poly)-phosphates were subsequently separated as above.

Data were plotted and curves analysed by Graph Pad Prism (Graph Pad Software Inc, San Diego, U.S.A.). Antagonist action was analysed by the generation of Schild plots according to Arunlakshana & Schild (1959). The pA_2 values when there was a single concentration of antagonist were calculated according to Furchgott (1972): $pA_2 = \log(DR-1) + p[A]$, where DR was the dose ratio at 50% of maximal response and p[A] was $-\log_{10}$ of the molar antagonist concentration. In some experiments the Schild plots had a slope different from 1, in which case the value of the intercept on the X axis was described as the apparent pA_2. In some cases the dose-response curves to agonists in the presence of suramin failed to reach a clear plateau, but did reach a level close to the maximum response seen in the absence of suramin. In these cases the response at the highest dose of agonist used was taken as the maximal response for the purposes of calculating the DR, and resultant pA_2 values are referred to as the apparent pA_2.

Where significance of differences are stated these were calculated by Student's *t* test.

Cell culture supplies were from GIBCO, Paisley, Scotland, except for foetal calf serum which was from Advanced Protein Products Ltd, West Midlands, U.K. *myo*-[2-^{3}H]-inositol was purchased from Amersham, Bucks, U.K. Drugs and compounds were from Sigma, Poole, U.K., except for 2-methylthioadenosine triphosphate (2MeSATP) from Research Biochemicals Inc., St Albans, U.K., PPADS from Tocris-Cookson Ltd, Bristol, U.K. and ARL 67156 which was a kind gift from Dr P. Leff, Astra Charnwood, Loughborough, U.K.

Results

We have shown in experiments preliminary to the present paper that the t-P_{2Y}-1321N1 cells respond to 2MeSATP but not UTP, the h-P_{2U}-1321N1 cells respond to UTP but not 2MeSATP, and that both cells respond to ATP, consistent with earlier observations (Parr *et al.*, 1994; Filtz *et al.*, 1994). Figure 1 shows the results of experiments investigating the effect of suramin on the response of the t-P_{2Y}-1321N1 cells to 2MeSATP. Suramin (10–100 μM) gave a parallel rightward shift of the concentration-response curves to 2MeSATP. On Schild analysis this generated a straight line with a slope not significantly different from unity (1.16 ± 0.08). The data in Figure 1 are pooled from 3 separate experiments; when each experiment was analysed separately the Schild plots gave pA_2 values of 5.77 ± 0.11.

When the same analysis was undertaken for the h-P_{2U}-1321N1 cells the effect of suramin was less marked (Figure 2). Suramin at 30 μM had no effect on the position of the concentration-response curve to UTP, and the rightward shift of the EC_{50} with 100 μM suramin was 4.56 ± 0.68 fold for the h-P_{2U}-1321N1 cells compared to 128.8 ± 15.4 for the t-P_{2Y}-1321N1 cells (significantly different at $P<0.001$). As expected from these data, the resulting Schild plot was less satisfactory (Figure 2b), giving an apparent pA_2 value of 4.32 ± 0.13 (from 3 separate experiments), but with a slope of 1.57 ± 0.19.

These results are consistent with the proposal that suramin shows some selectivity for the P_{2Y}-purinoceptor over the P_{2U}-purinoceptor. However, agonists such as 2MeSATP and UTP are known to be subject to degradation by ectonucleotidases, and suramin is known to be an ectonucleotidase inhibitor. It is therefore possible that the apparent effects of suramin as an antagonist are influenced by its action as an inhibitor. Differential effects of ectonucleotidase breakdown on the two agonists used here could therefore lead to an erroneous estimate of the selectivity of suramin as an antagonist. The effects of ectonucleotidase breakdown on the concentration-response curves reported, and on the effects of suramin, were investigated by the use of the ectonucleotidase inhibitor ARL 67156.

Figure 3 shows that the presence of ARL 67156 produced an elevation of the top and bottom of the concentration curve for 2MeSATP stimulation of t-P_{2Y}-1321N1 cells, and a small but insignificant shift of this curve to the left (log EC_{50} of -7.55 ± 0.17 in the absence of ARL 67156, and -8.08 ± 0.19 in the presence of 100 μM ARL 67156; data from 3 experiments). In the presence of suramin the concentration-response curves in the presence and absence of ARL 67156 were even closer (log EC_{50} values of -5.53 ± 0.10 in the absence, and

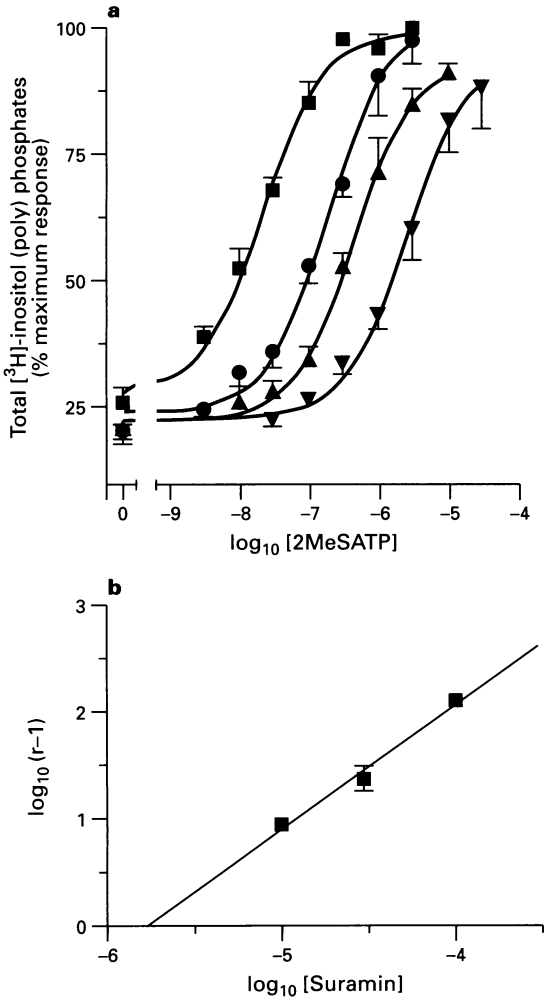

Figure 1 Effect of suramin on the response to 2MeSATP of P_{2Y}-transfected cells. (a) Concentration-response curves for 2MeSATP without suramin (■), and in the presence of suramin 10 μM (●), 30 μM (▲) and 100 μM (▼). (b) Schild plot of data from (a). Data (mean ± s.e.mean) are pooled from 3 separate experiments each undertaken in triplicate, and normalized as a % of the response to the highest dose of agonist, in the absence of suramin.

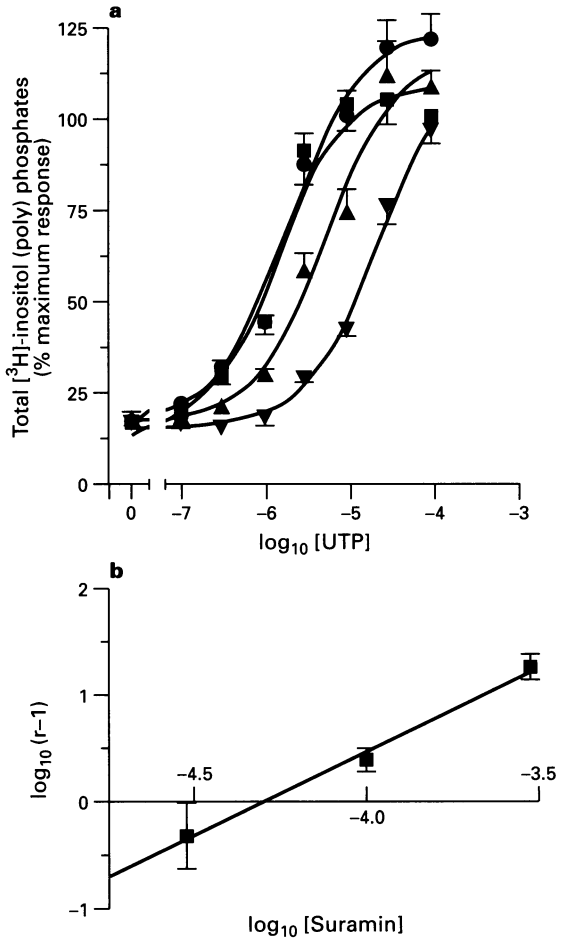

Figure 2 Effect of suramin on the response to UTP of P_{2U}-transfected cells. (a) Concentration-response curves for UTP without suramin (■), and in the presence of suramin 30 μM (●), 100 μM (▲) and 300 μM (▼). Schild plot of data from (a). Data (mean ± s.e.mean) are pooled from 3 separate experiments each undertaken in triplicate, and normalized as a % of the response to the highest dose of agonist, in the absence of suramin.

-5.79 ± 0.17 in the presence, of 100 μM ARL 67156; not significantly different). Suramin generated the expected shift to the right. In the absence of ARL 67156 the pA_2 values for suramin were 6.06 ± 0.40, and in the presence of ARL 67156 the apparent pA_2 values were 6.53 ± 0.27 ($n=3$; not significantly different).

Figure 4 shows results when the same protocol was applied to the h-P_{2U}-1321N1 cells stimulated with UTP. Again ARL 67156 raised the top and bottom of the concentration-response curves, and gave a small but insignificant shift of the curve to the left (log EC_{50} of -5.85 ± 0.06 in the absence, and -6.18 ± 0.13 in the presence of 100 μM ARL 67156). In the presence of suramin the ectonucleotidase inhibitor had no effect on the concentration-response curve to UTP. The apparent pA_2 values for suramin in the presence of ARL 67156 were 4.91 ± 0.16, compared to 4.72 ± 0.14 in its absence ($n=3$).

We have previously reported that PPADS is an effective antagonist at bovine aortic endothelial P_{2Y}-, but not P_{2U}-, purinoceptors (Brown *et al.*, 1995). We therefore investigated the effect of this antagonist at the cloned and transfected receptors. Figure 5 shows the influence of 3–30 μM PPADS on the response of t-P_{2Y}-1321N1 cells to 2MeSATP. Each concentration generated a shift in the curves to the right. For example, in one typical experiment the EC_{50} in the absence of PPADS was 0.024 μM, but in the presence of 3, 10 and 30 μM PPADS was 0.031, 0.085 and 0.260 respectively. Schild analysis gave data consistent with a straight line, with apparent pA_2 values of 5.98 ± 0.65, but with a slope of 0.55 ± 0.15 ($n=3$). Examination of data from individual experiments shows that there was a tendency for the presence of PPADS to cause the curves to be flatter at lower agonist concentrations but then to steepen, shifting the EC_{50} values to the right, but reaching the same maximal response at similar agonist concentrations. This and a slope of less than unity seen with the Schild analysis indicate that the effect of PPADS is not due to classical competitive antagonism.

In contrast to this characteristic effect of PPADS on the P_{2Y}-purinoceptor, there was no antagonist effect of this compound on the response of h-P_{2U}-1321N1 cells to UTP. This is illustrated in Figure 6, where it can be seen that the concentration-response curve to UTP was unaffected by the presence of 30 μM PPADS.

Discussion

In the rapidly expanding field of P_2-purinoceptor pharmacology, the paucity of selective antagonists acts as a considerable impediment to progress. Those antagonists which are available are poorly characterized with respect to actions at the different types of G protein-coupled P_2-purinoceptors. In this study we have set out to investigate the actions of two commonly uti-

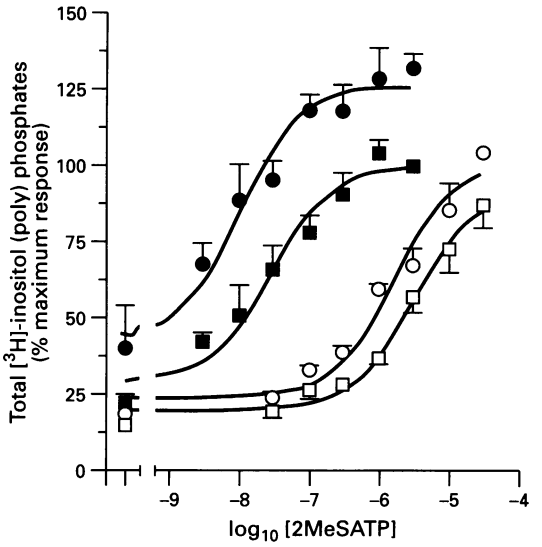

Figure 3 Effect of the ectonucleotidase inhibitor, ARL 67156 (100 μM) on the response of P_{2Y}-transfected cells to 2MeSATP in the presence and absence of 30 μM suramin. Concentration-response curve to 2MeSATP alone (■), 2MeSATP with ARL 67156 (●), 2MeSATP with suramin (□) and 2MeSATP with both suramin and ARL 67156 (○). Data are pooled from 3 separate experiments each in triplicate, normalized to % of the response to the highest concentration of agonist in the absence of added agents, and expressed as mean ± s.e.mean.

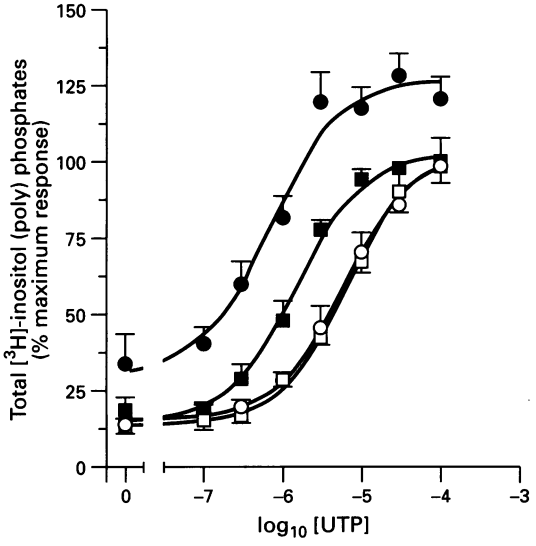

Figure 4 Effect of the ectonucleotidase inhibitor, ARL 67156 (100 μM), on the response of P_{2U}-transfected cells to UTP in the presence and absence of 30 μM suramin. Concentration-response curve to UTP alone (■), UTP with ARL 67156 (●), UTP with suramin (□) and UTP with both suramin and ARL 67156 (○). Data are pooled from 3 separate experiments each in triplicate, normalized to % of the response to the highest concentration of agonist in the absence of added agents, and expressed as mean ± s.e.mean.

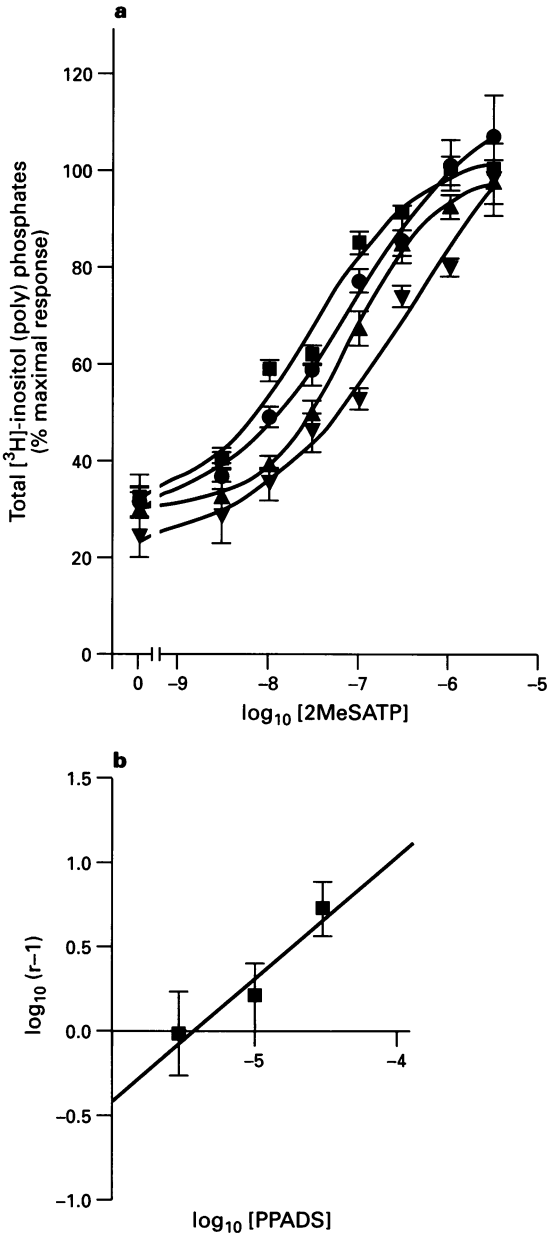

Figure 5 Effect of PPADS on the response to 2MeSATP of P_{2Y}-transfected cells. (a) Concentration-response curves for 2MeSATP without PPADS (■), and in the presence of PPADS 3 μM (●), 10 μM (▲) and 30 μM (▼). (b) Schild plot of data from (a). Data (mean ± s.e.mean) are pooled from 3 separate experiments each undertaken in triplicate, and normalized as a % of the response to the highest dose of agonist, in the absence of PPADS.

lized antagonists, suramin and PPADS, at cloned and transfected examples of two of the most widespread of these receptors, the P_{2Y}- and the P_{2U}-purinoceptors. The receptors are transfected into the same host cells, and so the comparison between the receptors is not contaminated by differences caused by the host cells. The P_{2U}-purinoceptors are cloned from human cDNA, while the P_{2Y}-purinoceptors are from turkey sequences, so it must be asked whether the differences are due to the species of origin, rather than the intrinsic receptor subtype. Review of the amino acid sequences reveals that the receptor types P_{2U} and P_{2Y} are each highly conserved across species, while the two receptor types show only selective and limited homology with each other. This is pertinently illustrated by the comparison of the putative G protein interactive region of the 3rd intracellular loop, and the putative ligand binding basic residues of the plasma membrane-extracellular interface of transmembrane domains 3, 6 and 7 (Erb *et al.*, 1995). In the 3rd intracellular loop there is essentially complete homology between bovine and turkey P_{2Y} sequences (Filtz *et al.*, 1994; Henderson *et al.*, 1995) but no homology between these and the murine and human P_{2U} sequences, which are themselves very close. Contrasting with this, the positively charged residues associated with ligand binding in the P_{2U} sequence (Erb *et al.*, 1995) are highly conserved both between species and between P_{2U}- and P_{2Y}-purinoceptors.

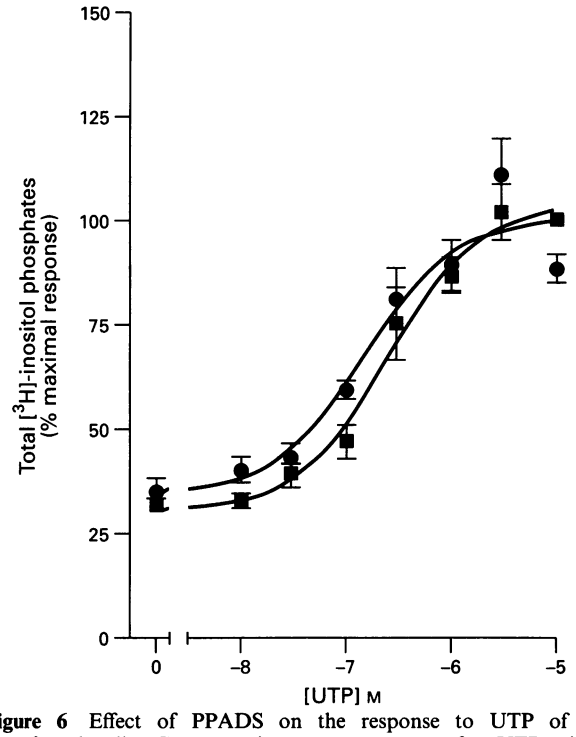

Figure 6 Effect of PPADS on the response to UTP of P_{2U}-transfected cells. Concentration-response curves for UTP without PPADS (■) and with 30 μM PPADS (●). Data (mean ± s.e.mean) are pooled from 3 separate experiments each undertaken in triplicate, and normalized as a % of the response to the highest dose of agonist, in the absence of PPADS.

Other residues in these same regions are however much more highly conserved across species of the same receptor type, than across receptor types. In summary, the very high degree of conservation across species in strategic domains of the same receptor indicate that the differences between the h-P_{2U}-1321N1 and t-P_{2Y}-1321N1 cells are due to the difference in receptor subtype transfected.

The results presented here show that suramin acts as a competitive antagonist at the recombinant P_{2Y}-purinoceptor, and that this is with a pA_2 value which is similar to that reported elsewhere for the antagonism by suramin of responses at P_{2X}-purinoceptors (e.g. Trezise *et al.*, 1994) as well as that reported for P_{2Y}-responses in, for example, bovine aortic endothelial cells (Wilkinson *et al.*, 1993; 1994) and the negatively linked adenylyl cyclase response in C6 glioma cells (Boyer *et al.*, 1994). In contrast with this, the response at the h-P_{2U}-1321N1 cells was relatively refractory to suramin, showing that suramin was not a potent antagonist at the cloned P_{2U}-purinoceptor. Data from experiments with higher concentrations of suramin (compare concentrations used in Figure 2a with those in Figure 1a) did generate a Schild plot with a straight line, which had a slope significantly different from unity, indicating that the low potency effect seen was not due to simple competitive antagonism. These conclusions are consistent with the results previously reported on the native P_{2U}-purinoceptor of bovine aortic endothelial cells (Wilkinson *et al.*, 1993; 1994). We saw no evidence of a reduced maximal response as reported by Boyer *et al.* (1994) with suramin and the P_{2Y}-purinoceptor responses of turkey erythrocyte membranes but this may have been due to non-receptor sites of action, such as at G proteins.

It is now apparent that the classification of P_2-purinoceptors, based on rank order of agonist potency, has been seriously compromised by differential ectonucleotidase breakdown (see Kennedy & Leff, 1995, for review). While most clearly documented for P_{2X}-purinoceptors, the same potential problem occurs with P_{2Y}- and P_{2U}-purinoceptors. Furthermore, when an antagonist is also known to be an effective ectonucleotidase inhibitor, which is the case for suramin (Hourani & Chown, 1989), then this could influence the apparent selectivity of the competitive antagonism. If UTP were more subject to removal by ectonucleotidases than 2MeSATP, then the enhancing of the extracellular concentrations of the agonist by suramin inhibition of breakdown would lead to an underestimate of its effectiveness as a competitive antagonist at the P_{2U}-purinoceptor. The results obtained here with the ectonucleotidase inhibitor, ARL 67156 (Crack *et al.*, 1995) show that this is not the case. The results do support the view that suramin is an effective inhibitor of ectonucleotidases: ARL 67156 has a small effect in the absence, but not in the presence, of suramin. Suramin is therefore an antagonist and an inhibitor, while ARL 67156 is solely an inhibitor. However, the effect of ectonucleotidase inhibition in the absence of antagonism (ARL 67156) is not significantly different between the 2 receptor subtypes; if anything it is slightly larger with the stimulation of t-P_{2Y}-1321N1 cells by 2MeSATP than with the stimulation of h-P_{2U}-1321N1 cells by UTP. The apparent pA_2 values for suramin are not significantly affected by the presence of ARL 67156 with either receptor-agonist system used, confirming the differential competitive antagonism of suramin at these two receptors.

There is no obvious explanation for the raising of the concentration-response curves by ARL 67156. If this is due to agonist activity of this compound (Crack *et al.*, 1995), then it is at a receptor not stimulated by either 2MeSATP or UTP, and at which suramin is an effective antagonist, since the raising of the curve is not seen in the presence of suramin. If it is due to the inhibition of ectonucleotidase, then it is raising the level of an endogenous nucleotide which is also acting at a receptor not activated by 2MeSATP or UTP.

PPADS, like suramin, was proposed as a selective competitive antagonist at P_{2X}-purinoceptors, but has since been shown to be effective at some P_{2Y}-purinoceptors (Boyer *et al.*, 1994; Windscheif *et al.*, 1994; 1995; Brown *et al.*, 1995). However, a degree of selectivity between putative different P_{2Y}-purinoceptors (Boyer *et al.*, 1994; Windscheif *et al.*, 1995) and a very clear lack of effect at bovine aortic endothelial P_{2U}-purinoceptors have been reported. The overall conclusion from the results presented here is that PPADS acts as an antagonist at the transfected P_{2Y}-purinoceptor, but not at the transfected P_{2U}-purinoceptor. Limited availability of ARL 67156 precluded undertaking a similar analysis of a possible influence of ectonucleotidase breakdown as with the suramin studies. However, the limited influence of ectonucleotidases revealed by these experiments, and the report that PPADS is much less effective than suramin as an inhibitor (Windscheif *et al.*, 1995), make it unlikely that inhibition of ectonucleotidase activity by PPADS has a substantial influence on the outcome of these investigations. The absence of any effect at P_{2U}-purinoceptors is consistent with that seen in the bovine aortic endothelial cell (Brown *et al.*, 1995), and in this respect the antagonist is highly selective for P_{2Y}- compared to P_{2U}-purinoceptors. However, two points must be considered. Firstly, the drug is an effective antagonist at P_{2X}-purinoceptors, and therefore has only limited selectivity. Secondly, the nature of the antagonism seen at P_{2Y}-purinoceptors is not clear. The shift in EC_{50} values for 2MeSATP with the t-P_{2Y}-1321N1 cells is clear evidence of antagonism, the maintenance of the maximum response suggests that it is not non-competitive, and the slope of the Schild plot indicates that it is not simple competitive antagonism. This is consistent with our earlier observations on bovine aortic endothelial cells, when we concluded that while the data show aspects of competitive antagonism, we could not exclude a non-competitive component (Brown *et al.*, 1995). There are previous indications of Schild analyses inconsistent with simple competitive antagonism by PPADS of P_{2Y}-purinoceptors (Windscheif *et al.*, 1995), and consequent difficulty in assigning unequivocal pA_2 values. The explanation available to these authors, of multiple native receptors, is not a possibility in our transfected system. Despite these difficulties, the generation of apparent pA_2 values equivalent to a concentration of about

1 μM indicates that PPADS is a relatively potent antagonist at these P_{2Y}-purinoceptors, showing a similar potency to that previously reported for P_{2X}-purinoceptors (e.g. Lambrecht *et al.*, 1992). Its complete lack of effect at P_{2U}-purinoceptors makes it a useful selective antagonist in investigations of G protein-coupled P_2-purinoceptors.

We thank the Wellcome Traust and the MRC for financial support, and Astra-Charnwood for the gift of ARL 67156.

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(Received December 14, 1995
Revised January 22, 1996
Accepted January 30, 1996)