British Journal of Pharmacology (1995) 116, 2413-2416

PPADS: an antagonist at endothelial P_{2Y}-purinoceptors but not P_{2U}-purinoceptors

Colin Brown, Bhavna Tanna & [1]Michael R. Boarder

Department of Cell Physiology and Pharmacology, Medical Sciences Building, University of Leicester, University Road, Leicester LE1 9HN

1 Bovine aortic endothelial (BAE) cells contain two co-existing receptors for extracellular ATP, the P_{2Y} and P_{2U}-purinoceptors. Here we have determined whether the proposed P_{2X}-purinoceptor antagonist, pyridoxalphosphate-6-azophenyl-2', 4'-disulphonic acid (PPADS) could distinguish between these two receptor subtypes.

2 Cells labelled with *myo*-[2-^{3}H]-inositol were stimulated with increasing concentrations of either the P_{2Y}-agonist, 2MeSATP, or the P_{2U}-agonist, UTP in the absence or presence of 30 μM PPADS. The accumulation of total [^{3}H]-inositol (poly)phosphates mediated by 2MeSATP was markedly attenuated by PPADS, whereas the response to UTP was not significantly affected.

3 Stimulation of BAE cells with increasing concentrations of ATP showed a reduced response in the presence of 10 μM PPADS, but this effect of the antagonist was not significant. By contrast, inhibition of the response to ADP was profound and highly significant.

4 These observations show that PPADS is not a selective P_{2X}-purinoceptor antagonist, but is able to distinguish between P_{2Y}- and P_{2U}-purinoceptors in BAE cells, and indicate that this compound may provide a useful tool in the study of multiple subtypes of P_2-purinoceptors. Furthermore the results are consistent with the hypothesis that ATP interacts with both receptor subtypes, but that the action of ADP is primarily at the P_{2Y}-purinoceptor in these endothelial cells.

Keywords: Purinoceptors; endothelial cells; inositol phosphate; antagonist; pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid

Introduction

The cell surface receptors for ATP and ADP, collectively classed as P_2-purinoceptors, are known to comprise a group of diverse G protein-coupled and ion channel receptors with widespread influence over bodily functions (Dubyak & El Moatassim, 1993). Progress in the study of these receptors has been restrained by the lack of cloned receptors and the unavailability of selective antagonists. Recently a number of reports of cloning of the P_2-purinoceptors have been published, including both ion channel P_{2X}-purinoceptors (Valera *et al.*, 1994; Brake *et al.*, 1994) and the G protein-coupled P_{2Y}-purinoceptors (Webb *et al.*, 1993; Filtz *et al.*, 1994) and P_{2U}-purinoceptors (Lustig *et al.*, 1993; Parr *et al.*, 1994). While these developments enable substantial further advances in this receptor field, the limited usefulness of antagonists is still a major problem. Several antagonists have been used successfully despite their restricted selectivity. Suramin for example, appears to have specific P_2-purinoceptor antagonist activity when used at high concentrations (Dunn & Blakely, 1988; Hoyle *et al.*, 1990; Leff *et al.*, 1990). First described as an antagonist at P_{2X}-purinoceptors, suramin is now known to be effective at P_{2Y}-purinoceptors, although it does exhibit a limited selectivity between the P_{2Y}- and the P_{2U}-purinoceptors of bovine aortic endothelial (BAE) cells (Wilkinson *et al.*, 1993; 1994). The use of reactive blue 2 (Cibacron blue) as a P_{2Y}-antagonist (Burnstock & Warland, 1987) is compromised by complex effects on the action of α,β-methylene ATP (an effective agonist at P_{2X}-purinoceptors) and non-selective effects at higher concentrations (e.g. Trezise *et al.*, 1994).

Recently a further candidate for selective competitive antagonist activity at P_{2X}-purinoceptors has emerged. Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) has been shown to act at the α,β-methylene binding site of P_{2X}-purinoceptors to effect antagonism in the low micromolar range (Lamprecht *et al.*, 1992; Ziganshin *et al.*, 1993) and has been used as a P_{2X}-antagonist in several further studies (e.g. Trezise *et al.*, 1994; Connolly *et al.*, 1995). In the present paper we show that PPADS is an effective antagonist, within this concentration-range, of the action of ATP on the P_{2Y}-purinoceptor of BAE cells, while remaining totally ineffective at the BAE cell P_{2U}-purinoceptor.

Methods

BAE cells were prepared by the method of Booyse *et al.*, (1975) and cultured in Minimum Essential Medium D-valine with 10% foetal calf serum, 10% new-born calf serum, 25 iu ml^{-1} penicillin, 25 μg ml^{-1} streptomycin, 10 mg ml^{-1} gentamycin, and 27 mg ml^{-1} glutamine in 95.5% air CO_2 at 37°C. The Factor VIII:R immunofluorescent cultures were used in 24 well plates.

Cells just about to reach confluence were labelled for 48 h with *myo*-[2-^{3}H]-inositol (1 μCi ml^{-1}, 0.5 ml per well) in M199 with glutamine, penicillin, streptomycin and fungizone at concentrations as above. Cells were then washed 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) and preincubated for 10 min in BSS with 10 mM LiCl followed by stimulation with agonists in BSS with LiCl but without $CaCl_2$. The total [^{3}H]-inositol (poly)phosphates were subsequently separated on small Dowex-1(Cl^-) columns.

Data were plotted by Fig P (Elsevier, Cambridge, U.K.), EC_{50} values were determined by Graph-Pad (Graph-Pad Software Inc, San Diego, U.S.A.), and statistical analysis of data was by 2-way analysis of variance of significance of difference between curves and by Student's *t* tests. Data are in each case presented as mean ± s.e.mean.

[1] Author for correspondence.

Results

We have previously shown that P_{2Y}- and P_{2U}-purinoceptors co-exist in the BAE cells, and that 2MeSATP and UTP respectively can be used as selective agonists at these two receptors, within the concentration-range used in the present study (Wilkinson *et al.*, 1993). Figure 1a shows a typical concentration-response curve to 2MeSATP, with stimulation occurring at 0.03 μM and a maximal response by 30 μM. In the presence of 30 μM PPADS the concentration-response curve to 2MeSATP was shifted to the right, with a minimally effective concentration of 2MeSATP of 3 μM. With 30 μM of the P_{2Y} agonist, the presence of 30 μM PPADS reduced the response to 30% of that seen in the absence of PPADS ($P<0.05$ by analysis of variance of curves constructed in the presence and absence of 30 μM PPADS and pooled across 3 separate experiments). A plateau could not reliably be obtained at this concentration of PPADS, preventing the generation of EC_{50} values pooled across experiments. When similar experiments

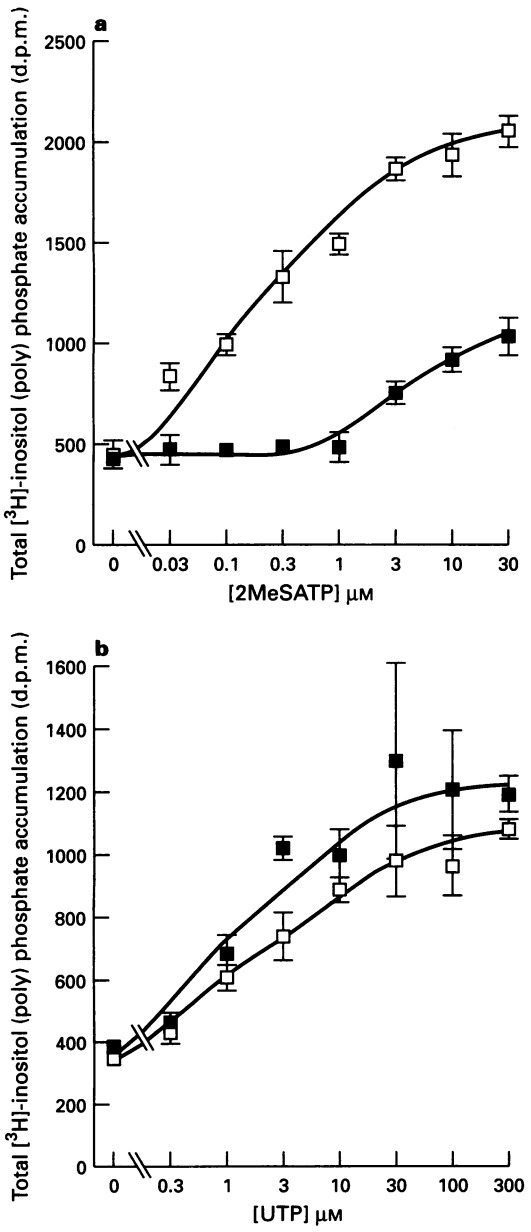

Figure 1 Concentration-effect curves for accumulation of total [^{3}H]-inositol (poly)phosphates in BAE cells stimulated for 15 min with increasing concentrations of (a) 2MeSATP, or (b) UTP, in the absence (□) or presence (■) of 30 μM PPADS. Data (d.p.m.) are mean ± s.e.mean (n = 3) from a single representative experiment (see text for data pooled across experiments).

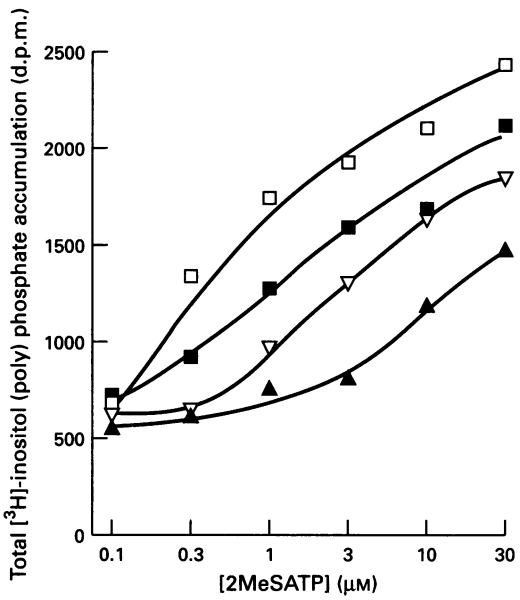

Figure 2 Concentration-effect curves for the accumulation of total [^{3}H]-inositol (poly)phosphates in BAE cells stimulated for 15 min with increasing concentrations of 2MeSATP in the absence (□) or presence of 1 μM (■), 3 μM (▽), or 10 μM (▲) PPADS. Data (d.p.m.) are mean from a single representative experiment performed in duplicate.

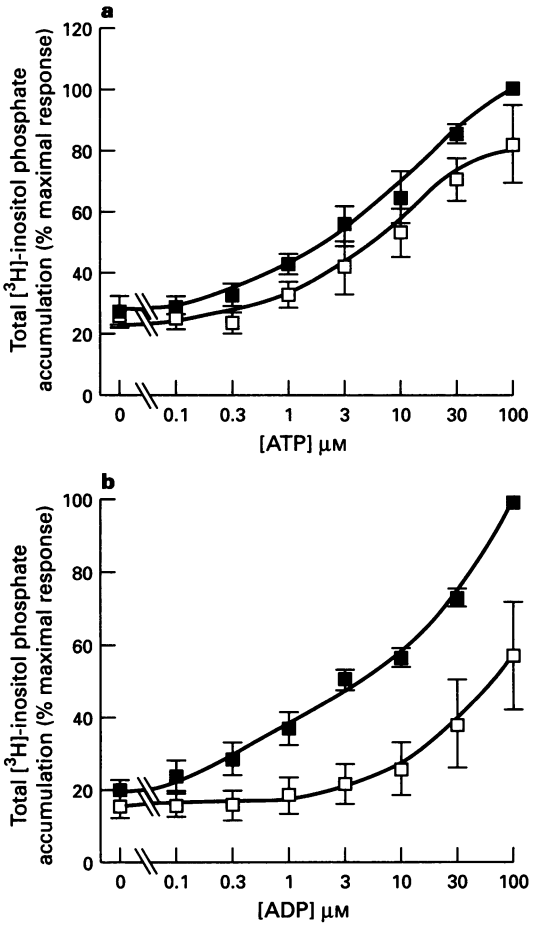

Figure 3 Concentration-effect curves for the accumulation of total [^{3}H]-inositol (poly)phosphates in BAE cells stimulated for 15 min with increasing concentrations of (a) ATP, or (b) ADP, in the absence (■) or presence (□) of 10 μM PPADS. Data (d.p.m.) are mean ± s.e.mean pooled across 4 separate experiments.

were undertaken with selective stimulation of the P_{2U}-purinoceptors by UTP, there was not attenuation of the response by PPADS (Figure 1b). Conversely there was an increase in the mean level of response to UTP in 3 out of the total 4 experiments undertaken. Pooled across these four experiments the EC_{50} of the response to UTP was 21.2 ± 10 μM in the absence of PPADS and 14.08 ± 6.3 μM in the presence of 30 μM PPADS (not significantly different), while in the presence of PPADS there was a non-significant increase in the maximal response to 137 ± 16.6% of that in its absence.

The data with 30 μM PPADS were consistent with the observation that there was a parallel shift to the right of the 2MeSATP response in the presence of the antagonist. The data in Figure 2 show an apparent parallel shift in the presence of 1–10 μM PPADS. However, it was not possible to construct reliable full dose-response curves in the presence of antagonist, perhaps in part due to difficulties with using 2MeSATP at the high concentrations which would be required, so a full quantitative analysis was not possible, and a reduction in maximal responses cannot be excluded.

The dominant endogenous agonists at these endothelial receptors are likely to be ATP and ADP, perhaps principally of platelet origin. To study the effect of PPADS on stimulation by these agonists we constructed concentration-response curves to ATP and ADP in the presence and absence of PPADS (Figure 3). The influence of PPADS on these responses was more complex. In each experiment undertaken PPADS caused both a shift in the ATP curve to the right and a reduction in the response to maximum concentration of agonist used (100 μM), but there was no significant difference between the curves on analysis of variance of data pooled across 4 experiments. Plateaus were not reliably seen in this series of experiments with either ADP or ATP. In each experiment with ADP as the agonist, PPADS caused a substantial flattening of the major part of the curve (e.g. Figure 3b). This time the effect of PPADS was significant ($P<0.0001$) on analysis of variance of curves pooled across the 4 experiments.

Discussion

The results presented show that PPADS acts as an antagonist at the P_{2Y}-purinoceptor of cultured BAE cells. The data shown here suggest that this is competitive antagonism, but the data do not exclude a non-competitive aspect of the action of PPADS. The data do unequivocally show that this was selective with respect to the two G protein-coupled P_2-purinoceptors found on BAE cells: PPADS is not an antagonist at this P_{2U}-purinoceptor. We have previously shown that suramin is able to distinguish between these two endothelial receptors. In both cultured cells and aortic collateral artery rings the responses to P_{2Y}-purinoceptor activation were subject to competitive antagonism by suramin in a manner not seen with responses to P_{2U}-purinoceptor activation (Wilkinson *et al.*, 1993; 1994). However, suramin was able to attenuate the P_{2U}-purinoceptor response, but with a lower potency and not in a clearly competitive manner. By contrast, in this study PPADS was able to produce a much clearer selective antagonism of the P_{2Y}-purinoceptor response.

These results suggest that PPADS is a useful tool in the investigation of P_2-purinoceptor responses, particularly where P_{2U}- and P_{2Y}-purinoceptors co-exist. This occurs not only in endothelial cells, but also for example in hepatocytes (Keppens & De Wulf, 1993). In bovine endothelial cells of the aorta and its collaterals we have presented data indicating that, while ATP is an effective agonist at both the P_{2Y}- and the P_{2U}-purinoceptors, the response to ADP is predominantly due to its action at the P_{2Y}-purinoceptor (Purkiss *et al.*, 1994; Wilkinson *et al.*, 1994). We have used the responses to UTP and 2MeSATP in the presence of PPADS to assess further this hypothesis. The response to ATP was partially inhibited by PPADS in each of the individual experiments undertaken, but this was not significant when the pooled data were analysed. A partial loss of response to ATP is the outcome expected if this agonist was acting on both classes of receptor and the response at the P_{2U}-purinoceptor was unaffected by the antagonist, while the response at the P_{2Y}-purinoceptor was shifted to the right, beyond the range of the maximal concentration used. While the overall effect in the data collected here was not significant, the results are consistent with a partial loss of response to ATP in the presence of PPADS. By contrast there was substantial and highly significant inhibition of the response to ADP by PPADS. The results clearly show a more profound effect of PPADS on the response to ADP than on the response to ATP. The data therefore provide further support for the hypothesis that while ATP acts on both receptors, the ADP response is dominated by action at the P_{2Y}-purinoceptor.

Earlier work has suggested that PPADS can be used as a selective antagonist at P_{2X}-purinoceptors (see Introduction). For example Windscheif *et al.* (1994) show that while P_{2X}-purinoceptor-mediated vasomotor responses of the rat mesentery were potently antagonized by PPADS, there were only weak antagonistic effects on P_{2Y}- and no effect on P_{2U}-purinoceptor-mediated responses. They conclude, therefore, that PPADS can be used as a selective antagonist in this preparation. However, the experimental data described in the present study show that PPADS cannot be used as a selective antagonist under other experimental conditions. In agreement with this, a paper published while this work was in progress indicated that PPADS was an effective antagonist at the turkey erythrocyte phospholipase C-linked, P_{2Y}-purinoceptor (Boyer *et al.*, 1994). However, the same paper showed that PPADS doe not act as an antagonist at the rat C6 glioma adenylyl cyclase-coupled P_{2Y}-purinoceptor. The results we provide here show that PPADS is a potent antagonist at mammalian phospholipase C-coupled, P_{2Y}-purinoceptors but that surprisingly, this effect is selective for the P_{2Y}- and not the P_{2U}-purinoceptor, and indicate that PPADS is a useful compound in the study of multiple subtypes of G protein-linked P_2-purinoceptors.

References

BOOYSE, F.M., SEDLAK, B.J. & RAFELSON, M.E. (1975). Culture of arterial endothelial cells: characterisation and growth of bovine aortic cells. *Thromb. Diath. Haemorrh.*, **34**, 825–839.

BOYER, J.L., ZOHN, I.E., JACOBSON, K.A. & HARDEN, T.K. (1994). Differential effects of P_2-purinoceptor antagonists in phospholipase C- and adenylyl cyclase-coupled P_{2Y}-purinoceptors. *Br. J. Pharmacol.*, **113**, 614–620.

BRAKE, A.J., WAGNEBACH, M.J. & JULIUS, D. (1994). New structural motif for ligand gated ion channels defined by an ionotropic ATP receptor. *Nature*, **371**, 519–523.

BURNSTOCK, G. & WARLAND, J.J.I. (1987). P_2-purinoceptors of two subtypes in the rat mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P_{2Y}- but not the P_{2X}-purinoceptor. *Br. J. Pharmacol.*, **90**, 383–391.

CONNOLLY, G.P. (1995). Differentiation by pyridoxal 5-phosphate, PPADS and IsoPPADS between responses mediated by UTP and those evoked by α,β-methylene-ATP on rat sympathetic ganglia. *Br. J. Pharmacol.*, **114**, 727–731.

DUBYAK, G.R. & EL MOATASSIM, C. (1993). Signal transduction via P_2 purinergic receptors for extracellular ATP and other nucleotides. *Am. J. Physiol.*, **265**, C577–C606.

DUNN, P. & BLAKELY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

FILTZ, T.A., LI, Q., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1994). Expression of a cloned P_{2Y}-purinergic receptor that couples to phospholipase C. *Mol. Pharmacol.*, **46**, 8–14.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonises responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99**, 617–621.

KEPPENS, S. & DE WULF, H. (1993). The complex interaction of ATP and UTP with isolated hepatocytes. How many receptors? *Gen. Pharmacol.*, **24**, 283–289.

LAMPRECHT, G., FREIBE, T., GRIMM, U., WINDSCHEIF, U., BUNGARDT, E., HILDEBRANDT, C., BAUMERT, H.G., SPATZ-KUMBEL, G. & MUTSCHLER, E. (1992). PPADS, a novel functionally selective antagonist of P_2 purinoceptor mediated responses. *Eur. J. Pharmacol.*, **217**, 217–219.

LEFF, P., WOOD, B.E. & O'CONNOR, S.E. (1990). Suramin is a slowly-equilibrating but competitive antagonist at P_{2X}-receptors in the rabbit isolated ear artery. *Br. J. Pharmacol.*, **101**, 645–649.

LUSTIG, K.D., SHIAU, A.S., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 5113–5117.

PARR, C.E., SULLIVAN, D.M., PARADISO, A.M., LAZAROWSKI, E.R., BURCH, L.H., OLSEN, J.C., ERB, L., WEISMAN, G.A., BOUCHER, R.C. & TURNER, J.T. (1994). Cloning and expression of a human P_{2U} nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 3275–3279.

PURKISS, J.R., WILKINSON, G.F. & BOARDER, M.R. (1994). Differential regulation of inositol 1,4,5-trisphosphate by co-existing P_{2Y}-purinoceptors and nucleotide receptors on bovine aortic endothelial cells. *Br. J. Pharmacol.*, **111**, 723–728.

TREZISE, D.J., KENNEDY, I. & HUMPHREY, P.P.A. (1994). The use of antagonists to characterise the receptors mediating depolarisation of the rat isolated vagus nerve by α,β-methylene adenosine 5′-trisphosphate. *Br. J. Pharmacol.*, **112**, 282–288.

VALERA, S., HUSSY, N., EVANS, R.J., ADAMI, N., NORTH, R.A., SUPRENANT, A. & BUELL, G. (1994). A new class of ligand gated ion channel defined by P_{2X} receptor for extracellular ATP. *Nature*, **371**, 516–519.

WEBB, T.E., SIMON, J., KRISHEK, B.J., BATESON, A.N., SMART, T.G., KING, B.F., BURNSTOCK, G. & BARNARD, E.A. (1993). Cloning and functional expression of a brain G-protein coupled ATP receptor. *FEBS Lett.*, **324**, 219–225.

WILKINSON, G.F., MCKECHNIE, K., DAINTY, I.A. & BOARDER, M.R. (1994). P_{2Y}-purinoceptor and nucleotide receptor-induced relaxation of precontracted bovine aortic collateral artery rings: differential sensitivity to suramin and indomethacin. *J. Pharmacol. Exp. Ther.*, **268**, 881–887.

WILKINSON, G.F., PURKISS, J.R. & BOARDER, M.R. (1993). The regulation of aortic endothelial cells by purines and pyrimidines involves co-existing P_{2Y}-purinoceptors and nucleotide receptors linked to phospholipase C. *Br. J. Pharmacol.*, **108**, 689–693.

WINDSCHEIF, U., RALEVIC, V., BAUMERT, H.G., MUTSCHLER, E., LAMPRECHT, G. & BURNSTOCK, G. (1994). Vasoconstrictor and vasodilator responses to various agonists in the rat perfused mesenteric arterial bed: selective inhibition of PPADS of contractions mediated via P_{2X}-purinoceptors. *Br. J. Pharmacol.*, **113**, 1015–1021.

ZIGANSHIN, A.U., HOYLE, C.H.V., BO, X., LAMPRECHT, G., MUTSCHLER, E., BAUMERT, H.G. & BURNSTOCK, G. (1993). PPADS selectively antagonises P_{2X}-purinoceptor-mediated responses in the rabbit urinary bladder. *Br. J. Pharmacol.*, **110**, 1491–1495.

(Received February 27, 1995
Revised June 15, 1995
Accepted July 5, 1995)