British Journal of Pharmacology (1996) 118, 1806-1812

A comparison of the binding characteristics of recombinant P_{2X1} and P_{2X2} purinoceptors

[1]Anton D. Michel, *Kenneth Lundström, †Gary N. Buell, †Annmarie Surprenant, †Soledad Valera & Patrick P.A. Humphrey

Glaxo Institute of Applied Pharmacology, Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QJ; *Glaxo Research and Development, Gunnels Wood Rd, Stevenage, Herts, SG1 2NY and †Glaxo Institute for Molecular Biology, 14 chemin des Aulx, 1228 Plan-les-Ouates, Geneva, Switzerland

1 We have recently provided evidence that [^{35}S]-adenosine 5'-O-[3-thiotriphosphate] ([^{35}S]-ATPγS) can label the human bladder recombinant P_{2X1} purinoceptor (human P_{2X1} purinoceptor). In this study we have characterized the binding of [^{35}S]-ATPγS to a second P_{2X} purinoceptor subtype, the rat PC12 phaeochromocytoma cell recombinant P_{2X2} purinoceptor (rat P_{2X2} purinoceptor), and compared its binding properties with those of both endogenous and recombinant P_{2X1} purinoceptors.

2 Infection of CHO-K1 cells with the rat P_{2X2} purinoceptor using Semliki forest virus (SFV) resulted in the expression of high affinity ($pK_d = 9.3$; $B_{max} = 18.1$ pmol mg^{-1} protein) binding sites for [^{35}S]-ATPγS but not for [^{3}H]-α,β-methylene ATP ([^{3}H]-αβmeATP). Since functional P_{2X} purinoceptors could be detected electrophysiologically in these cells, but not in non-infected or CHO-K1 cells infected with SFV containing the LacZ gene, these results suggest that the rat P_{2X2} purinoceptor can be labelled using [^{35}S]-ATPγS.

3 The binding characteristics of the rat P_{2X2} purinoceptor were compared with those of the human P_{2X1} purinoceptor, which was also expressed in the CHO-K1 cells using SFV. A major difference between the two recombinant P_{2X} purinoceptor types was in the binding characteristics of α,β-methylene ATP (αβmeATP). Thus, in the absence of divalent cations, αβmeATP possessed low affinity for both the human P_{2X1} purinoceptor ($pIC_{50} = 7.2$) and rat P_{2X2} purinoceptor ($pIC_{50} = 7.1$) labelled using [^{35}S]-ATPγS. However, when the recombinant P_{2X} purinoceptors were labelled with [^{3}H]-αβmeATP in the presence of 4 mM $CaCl_2$, the affinity of αβmeATP for the human P_{2X1} purinoceptor increased (pIC_{50} for αβmeATP = 8.2), while the affinity of the rat P_{2X2} purinoceptor for αβmeATP did not change (pIC_{50} for αβmeATP = 6.8).

4 Affinity estimates of 15 other nucleotide analogues for the [^{35}S]-ATPγS binding sites on the two recombinant P_{2X} purinoceptor subtypes were surprisingly similar (less than 5 fold difference), the only exception being 2'-deoxy ATP which possessed 8 fold higher affinity for rat P_{2X2} than for human P_{2X1} purinoceptors. In contrast dextran sulphate and the P_2 purinoceptor antagonists, pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid and 4,4'-diisothiocyanatostilbene-2,2'disulphonic acid, possessed 7 to 33 fold higher affinity for the human P_{2X1} than for the rat P_{2X2} purinoceptor. These data provide a correlation coefficient (r) of 0.894.

5 There was some evidence for species differences in the P_{2X1} purinoceptor. Thus, most nucleotides possessed slightly greater (up to 9-10 fold), while the P_2 purinoceptor antagonists possessed slightly lower (up to 7-16 fold), affinity for the endogenous rat vas deferens and rat bladder P_{2X1} purinoceptors than for the human recombinant P_{2X1} purinoceptor. These differences were reflected in a slightly lower correlation coefficient, when comparing across species between the human recombinant P_{2X1} purinoceptor and the endogenous P_{2X1} purinoceptors labelled in either the rat deferens ($r = 0.915$) or the rat bladder ($r = 0.932$), than when comparing within species between the endogenous rat vas deferens and rat bladder P_{2X1} purinoceptors ($r = 0.995$).

6 In summary, [^{35}S]-ATPγS can be used to label the recombinant P_{2X1} and P_{2X2} purinoceptors. Despite the marked differences reported between these two forms of P_{2X} purinoceptor in functional studies, the differences in binding studies were more limited. However, a number of antagonists could discriminate between the P_{2X} purinoceptor subtypes in the binding studies raising expectations that selective antagonists for these receptors can be developed.

Keywords: P_{2X1} purinoceptor; P_{2X2} purinoceptor; PC12 phaeochromocytoma; [^{35}S]-adenosine 5'-O-(3-thiotriphosphate); [^{3}H]-α,β-methylene ATP; rat vas deferens; rat bladder; Semliki forest virus; CHO-K1 cell

Introduction

The P_2 purinoceptors which mediate the cellular effects of extracellular ATP are heterogeneous with at least five clearly defined subtypes being described (Fredholm *et al.*, 1994). Of this family, the P_{2X} purinoceptor subtype was originally defined by Burnstock & Kennedy, (1985), as possessing higher affinity for α,β-methylene ATP (αβmeATP) than for 2-methylthio ATP (2-meS-ATP). More recently, however, it has been suggested that the P_{2X} purinoceptor should be defined by structural criteria, since P_{2X} purinoceptors are ligand-gated cation channels and, as such, are distinct from the other P_2 purinoceptors which, structurally, appear to belong to the ever increasing family of seven transmembrane domain, G-protein coupled, receptors (Abbracchio & Burnstock, 1994).

While the definition of P_{2X} purinoceptors as ligand-gated cation channels simplifies the classification of P_2 purinoceptor subtypes it is necessary to consider further subdivisions within the P_{2X} purinoceptor class to accommodate data from both functional and molecular cloning studies (see Humphrey *et al.*,

[1] Author for correspondence.

1995). Thus, there is considerable evidence from functional studies for the existence of receptor heterogeneity since different rank orders of agonist potency have been reported at the ATP-gated channels present in a number of cell types. In myocytes isolated from rat bladder (Inoue & Brading, 1990), ATP and $\alpha\beta$meATP are approximately equipotent as agonists. Similarly, in studies on single cells isolated from rat vas deferens (Khakh *et al.*, 1995b), rat blood vessels (Evans & Kennedy, 1995), rat nodose ganglion (Khakh *et al.*, 1995a) and rat coeliac ganglion (Khakh *et al.*, 1995a), $\alpha\beta$meATP, 2-meS-ATP and ATP are of equal potency. In contrast, at the ATP-gated cation channels of rat SCG (Khakh *et al.*, 1995a) and PC12 phaeochromocytoma cells (PC12 cells, Brake *et al.*, 1994), 2-meS-ATP and ATP are considerably more potent than $\alpha\beta$meATP.

These data suggest the presence of both $\alpha\beta$meATP-sensitive and -insensitive P_{2X} purinoceptor subtypes. Furthermore, it is probable that the $\alpha\beta$meATP-sensitive P_{2X} purinoceptors are also heterogeneous. Thus, the P_{2X} purinoceptors present in smooth muscle of guinea-pig bladder (Inoue & Brading, 1990), rat vas deferens (Khakh *et al.*, 1995b) and rat blood vessels (Evans & Kennedy, 1994) show marked desensitization with repeated agonist application and rapid fade of response in the presence of agonist, while in nodose and coeliac ganglia desensitization is less pronounced and responses show little evidence of fade in the continued presence of agonist (Khakh *et al.*, 1995a). Furthermore, the $\alpha\beta$meATP-sensitive P_{2X} purinoceptors in smooth muscle and neurones can be differentiated by L-β,γ-methylene ATP (L-$\beta\gamma$meATP) which is a potent agonist on the smooth muscle form of P_{2X} purinoceptor but possesses low or no affinity for the functional P_{2X} purinoceptor type(s) in rat vagus nerve (Trezise *et al.*, 1995).

Molecular cloning studies have now provided definitive evidence for P_{2X} purinoceptor subtypes with the recent isolation of cDNA encoding ATP-gated cation channels from rat vas deferens, PC12 cells, human bladder, sensory neurones and brain (Brake *et al.*, 1994; Valera *et al.*, 1994; 1995; Lewis *et al.*, 1995; Chen *et al.*, 1995; Bo *et al.*, 1995). The P_{2X} purinoceptors from bladder and vas deferens are similar at the amino acid level, exhibiting up to 89% identity, suggesting that they may be species variants of the same receptor (Valera *et al.*, 1995), now referred to as the P_{2X1} purinoceptor (Lewis *et al.*, 1995). In contrast, the P_{2X} purinoceptor from PC12 cells, now defined as the P_{2X2} purinoceptor (Lewis *et al.*, 1995), exhibits only 41% identity with the vas deferens P_{2X1} purinoceptor (Valera *et al.*, 1994). The pharmacological properties of the recombinant receptors also differ, the P_{2X1} purinoceptors (Valera *et al.*, 1994; 1995) and the P_{2X3} purinoceptors isolated from sensory ganglia (Lewis *et al.*, 1995; Chen *et al.*, 1995) are sensitive to $\alpha\beta$meATP and desensitize rapidly, while the P_{2X2} purinoceptor isolated from PC12 cells (Brake *et al.*, 1994) and the P_{2X4} purinoceptor isolated from rat brain (Bo *et al.*, 1995) do not undergo marked desensitization and are insensitive to $\alpha\beta$ meATP.

Recent studies have shown that, under appropriate experimental conditions, it is possible to label directly the human bladder recombinant P_{2X1} purinoceptor (human P_{2X1} purinoceptor) using the radioligand [^{35}S]-adenosine 5'-O-thiotriphosphate ([^{35}S]-ATPγS) and that the binding characteristics of this recombinant P_{2X1} purinoceptor are very similar to those of the endogenous P_{2X1} purinoceptor of rat vas deferens (Michel *et al.*, 1996). With the recent cloning of the P_{2X2} purinoceptor subtype from rat PC12 cells, the aim of the present study was to determine first if the PC12 recombinant P_{2X2} purinoceptor (rat P_{2X2} purinoceptor) could also be directly labelled and, since this proved possible, to compare the binding characteristics of the human recombinant P_{2X1} and rat P_{2X2} purinoceptors. For these studies the recombinant P_{2X} purinoceptors were expressed using the Semliki forest virus (SFV) expression system since this viral infection procedure results in high levels of receptor expression (Lundström *et al.*, 1994), and has been successful for expression of the human recombinant P_{2X1} purinoceptor (Michel *et al.*, 1996).

Methods

Infection of CHO-K1 cells with P_{2X} purinoceptors

Infection of CHO-K1 cells was performed as described previously (Michel *et al.*, 1996). Briefly, the coding regions of the human P_{2X1} or rat P_{2X2} purinoceptor were amplified by PCR and cloned into the BamH1 site of the pSFV1 vector (Liljeström & Garoff, 1991; Lundström *et al.*, 1994). *In vitro* transcripts of these plasmids and the pSFV-Helper2 plasmid were prepared and electroporated into baby hamster kidney (BHK) cells according to Lundström *et al.* (1994) and the resulting virus stocks were collected 24 h later. CHO-K1 cells, grown as a monolayer culture, were infected with SFV, containing either the human bladder P_{2X1} purinoceptor (SFV-human P_{2X1}) or the rat PC12 cell P_{2X2} purinoceptor (SFV-rat P_{2X2}), at a multiplicity of infection of 10 and were harvested 16 h later by incubation in divalent cation-free PBS. In some studies CHO-K1 cells were infected with SFV in which the coding region for the LacZ gene was inserted in place of the P_{2X} purinoceptor coding region (SFV-LacZ). The infected and non-infected CHO-K1 cells used in this study were also examined by whole cell-voltage clamp recording techniques and those infected with either SFV-human P_{2X1} or SFV-rat P_{2X2} were shown to possess an ATP-gated cation channel while the non-infected and SFV-LacZ infected cells did not (data not shown).

Receptor binding studies

Washed membranes were prepared from non-infected and infected CHO-K1 cells as described previously (Michel *et al.*, 1996), while rat bladder membranes were prepared by the procedure described for the preparation of rat vas deferens membranes (Michel & Humphrey, 1994). The binding of [^{35}S]-ATPγS was examined using a 50 mM Tris, 1 mM EDTA assay buffer, pH 7.4 at 4°C, since [^{35}S]-ATPγS can only be used to label P_{2X} purinoceptors in the absence of divalent cations (Michel & Humphrey, 1996). When [^{3}H]-$\alpha\beta$meATP was the radioligand, 4 mM $CaCl_2$ was included in the assay buffer since calcium is required for high affinity binding of [^{3}H]-$\alpha\beta$meATP to its sites in rat vas deferens (Michel & Humphrey, 1993; 1994).

Incubations were performed at 4°C in a final assay volume of 250 μl for 3 h, in studies using [^{35}S]-ATPγS, or 40 min when [^{3}H]-$\alpha\beta$meATP was the radioligand. The 3 h incubation period in the studies using [^{35}S]-ATPγS was necessary to ensure that steady state levels of binding were achieved at each receptor studied. Thus, steady state levels of [^{35}S]-ATPγS binding (0.15 nM) to the recombinant P_{2X1} and P_{2X2} purinoceptors were attained within 40 min and were stable for at least a further 4 h. In studies on the endogenous P_{2X1} purinoceptors, equilibrium was only attained after 120–150 min. Reactions were terminated by vacuum filtration using either a Brandell 48 well or Packard Filtermate cell harvester and bound radioligand was determined by liquid scintillation spectrophotometry using either a Canbera Packard Topcount or 2200CA scintillation counter. Non-specific binding (NSB) of [^{35}S]-ATPγS was defined by use of either 300 nM 2-meS-ATP or 10 μM ATPγS, which gave essentially similar results. NSB of [^{3}H]-$\alpha\beta$meATP was defined using 10 μM L-$\beta\gamma$meATP. These concentrations of 2-meS-ATP and L-$\beta\gamma$meATP selectively inhibit high affinity binding of the radioligands to the recombinant human P_{2X1} purinoceptor but produce minimal inhibition of their binding to low affinity radioligand binding sites which may be present in some cell types and membrane preparations (Michel *et al.*, 1996).

In competition studies, the ability of a series of compounds to compete for the binding sites labelled by 0.15 nM [^{35}S]-ATPγS was determined over a range of concentrations spanning at least five log units. In saturation studies total binding and non specific binding was measured for 0.05–5 nM [^{35}S]-ATPγS, in triplicate and duplicate, respectively.

Data analysis

Saturation and competition binding data were analysed by iterative curve fitting procedures (Michel Humphrey, 1994). To enable ready comparison with data from our previous studies, the IC_{50} values determined in this study were not adjusted to take account of the presence of radioligand and are presented as the negative logarithm of the IC_{50} (pIC_{50}). Caveats about the calculation of equilibrium dissociation constants from such data have already been discussed (Michel & Humphrey, 1993; 1996). However, on the basis of the affinity estimates for [^{35}S]-ATPγS at the rat P_{2X2} purinoceptor ($pK_d = 9.3$; see Results) and human P_{2X1} purinoceptor ($pK_d = 9.1$; Michel *et al.*, 1996) it can be calculated, using the Cheng-Prussof equation (Cheng & Prussof, 1973), that the IC_{50} values will be only 1.31 and 1.18 fold greater, respectively, than the K_i. The pIC_{50} values given in this study are therefore considered to represent reasonable estimates of affinity. Unless otherwise stated the data presented are the mean ± s.e.mean of between 3 and 5 separate experiments.

Materials

The sources of 2-meS-ATP, L-βγmeATP, ATPγS, αβmeATP, α,β-methylene ADP (αβmeADP), ADP, ATP and β,γ-methylene ATP (βγmeATP) were as described previously (Michel & Humphrey, 1996). In addition 2-chloro-ATP (2Cl-ATP), adenosine 5'-tetraphosphate (APPPP), diadenosine pentaphosphate (AP5A), 2'-and3'-O-(4-benzoylbenzoyl)-ATP (dibenzyl-ATP), 2'-deoxy ATP (deoxy-ATP), 5' adenylylimidodiphosphate (βγimATP), (pyridoxal 5'-phosphate (P5P), cibacron blue, (+)-tubocurarine chloride and 4,4'-diisothiocyanatostilbene-2,2'disulphonic acid (DIDS) were obtained from Sigma. Suramin was generously supplied by Bayer while pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) was purchased from Cookson chemicals. Dextran sulphate (mol.wt. = 1500 approx) and adenosine-5'-O-(1-thiotriphosphate) (ATPαS) were from Calbiochem. The radioligands [^{35}S]-ATPγS (Specific activity 1500 Ci mmol^{-1}) and [^{3}H]-αβ meATP (Specific activity 28 Ci mmol^{-1}) were obtained from Amersham, UK.

Results

Characterization of [^{35}S]-ATPγS binding to CHO-K1 cells transiently expressing the recombinant rat P_{2X2} purinoceptor

Non-infected CHO-K1 cells possessed a low density of [^{35}S]-ATPγS binding sites ($pK_d = 8.7 \pm 0.1$; $B_{max} = 358 \pm 139$ fmol mg^{-1} protein; Michel *et al.*, 1996). However, following infection of these cells with the rat P_{2X2} purinoceptor using SFV, considerably higher levels of [^{35}S]-ATPγS binding were detected ($pK_d = 9.3 \pm 0.1$; $B_{max} = 18.1 \pm 1.5$ pmol mg^{-1} protein). In addition, the characteristics of the binding sites labelled by [^{35}S]-ATPγS in the CHO-K1 cells infected with the rat recombinant P_{2X2} purinoceptor using SFV were very different from those of the non-infected cells (cf. data from Table 1 with that from Michel *et al.*, 1996).

Binding studies with [^{3}H]-αβmeATP

We have previously shown that, in the presence of 4 mM $CaCl_2$, [^{3}H]-αβmeATP can label the recombinant P_{2X1} purinoceptor (Michel *et al.*, 1996). When examined in a 50 mM Tris 1 mM EDTA assay buffer supplemented with 4 mM $CaCl_2$ there were, however, only low levels of specific [^{3}H]-αβmeATP binding to membranes from both non-infected CHO-K1 cells and CHO-K1 cells infected with the rat recombinant P_{2X2} purinoceptor using SFV (2.2 ± 0.9 and 3.1 ± 0.5 d.p.m. μg^{-1} protein, respectively; $n = 3$; 1 nM [^{3}H]-αβmeATP). The low level of [^{3}H]-αβmeATP binding to the 'rat recombinant P_{2X2} purinoceptor' infected CHO-K1 cells, contrasts with the much higher levels of specific [^{3}H]-αβmeATP binding identified in CHO-K1 cells expressing the human recombinant P_{2X1} purinoceptor (102 ± 7 d.p.m. μg^{-1} protein; $n = 3$; 1 nM [^{3}H]-αβ-meATP; Michel *et al.*, 1996).

As a result of the low levels of binding and the low specific activity of the radioligand it was not possible to perform saturation experiments with [^{3}H]-αβmeATP on the rat recombinant P_{2X2} purinoceptor. However, in competition studies, αβmeATP competed with low affinity ($pIC_{50} = 6.8 \pm 0.3$;

Table 1 Competition binding studies at recombinant P_{2X} purinoceptors labelled with [^{35}S]-ATPγS

Compound	P_{2X1} purinoceptor pIC_{50}	P_{2X1} purinoceptor Hill slope	P_{2X2} purinoceptor pIC_{50}	P_{2X2} purinoceptor Hill Slope
ATP	8.93±0.05	1.03±0.04	8.92±0.07	0.94±0.05
ATPγS	8.08±0.03	0.87±0.07	8.70±0.05	1.03±0.04
2-me-S-ATP	8.23±0.14	0.80±0.06	8.68±0.04	0.89±0.11
αβmeATP	7.17±0.09	0.91±0.06	7.07±0.10	0.98±0.06
ADP	7.27±0.04	0.92±0.14	7.32±0.05	0.93±0.05
βγmeATP	6.25±0.08	0.84±0.09	6.66±0.05	0.92±0.04
L-βγmeATP	5.39±0.04	0.65±0.07	5.19±0.06	0.87±0.10
αβmeADP	4.22±0.04	0.75±0.13	4.27±0.11	0.80±0.01
2-Cl-ATP	8.80±0.02	0.62±0.06	8.45±0.03	0.97±0.03
APPPP	8.29±0.04	0.79±0.06	8.33±0.05	1.06±0.06
ATPαS	8.89±0.02	0.75±0.08	8.66±0.03	0.87±0.04
AP5A	6.81±0.05	1.02±0.13	7.23±0.01	1.03±0.04
βγimATP	7.20±0.04	0.98±0.11	6.95±0.05	1.05±0.03
Dibenzyl-ATP	8.22±0.11	0.77±0.10	7.99±0.02	0.89±0.11
Deoxy-ATP	6.56±0.12	0.86±0.14	7.44±0.01	0.90±0.07
Dextran	6.90±0.06	0.59±0.01	5.38±0.05	0.72±0.03
DIDS	5.35±0.02	1.27±0.15	3.97±0.07	0.62±0.03
PPADS	4.74±0.08	0.76±0.11	3.92±0.03	0.91±0.07
PPO_4	3.86±0.05	0.76±0.09	3.59±0.02	0.94±0.01
Cib blue	6.22±0.09	2.56±0.85	6.33±0.05	1.49±0.17
Suramin	6.47±0.09	0.94±0.07	6.16±0.02	0.85±0.06

Competition binding studies on the sites labelled by 0.15 nM [^{35}S]-ATPγS in membranes prepared from CHO-K1 cells infected, using Semliki Forest virus, with either the human recombinant P_{2X1} purinoceptor or the rat PC12 cell recombinant P_{2X2} purinoceptor. The data are the mean ± s.e.mean of $n = 3-5$ determinations. The data for ATP, ATPγS, 2-meS-ATP, αβmeATP, ADP, βγmeATP, L-βγmeATP and αβmeADP at the human recombinant P_{2X1} purinoceptor are from Michel *et al.* (1996).

$n_H = 0.85 \pm 0.08$; $n = 3$) for the [^{3}H]-$\alpha\beta$meATP binding sites labelled in the CHO-K1 cells infected with the rat recombinant P_{2X2} purinceptors using SFV.

Comparison of the recombinant human P_{2X1} and rat P_{2X2} purinoceptors labelled with [^{35}S]-ATPγS

Affinity estimates (pIC_{50} values) for a number of compounds were determined at the human recombinant P_{2X1} and rat recombinant P_{2X2} purinoceptors expressed in CHO-K1 cells. The affinity estimates for most nucleotide analogues, including $\alpha\beta$-meATP, were almost identical at the human recombinant P_{2X1} and rat recombinant P_{2X2} purinoceptors, although those for ATPγS, 2-meS-ATP and deoxyATP were 3 to 8 fold greater at the rat recombinant P_{2X2} purinoceptor than at the human recombinant P_{2X1} purinoceptor (see Table 1 and Figure 1a). Affinity estimates for the P_2 purinoceptor antagonists, PPADS and DIDS, and for dextran sulphate differed between the recombinant receptors being 7 to 33 fold greater at the human recombinant P_{2X1} purinoceptor than at the rat recombinant P_{2X2} purinoceptor. A comparison of affinity estimates for the 21 compounds examined at the human recombinant P_{2X1} and rat recombinant P_{2X2} purinoceptors produced a correlation coefficient of 0.894. (+)-Tubocurarine (up to 100 μM) did not inhibit [^{35}S]-ATPγS binding to either recombinant P_{2X} purinoceptor.

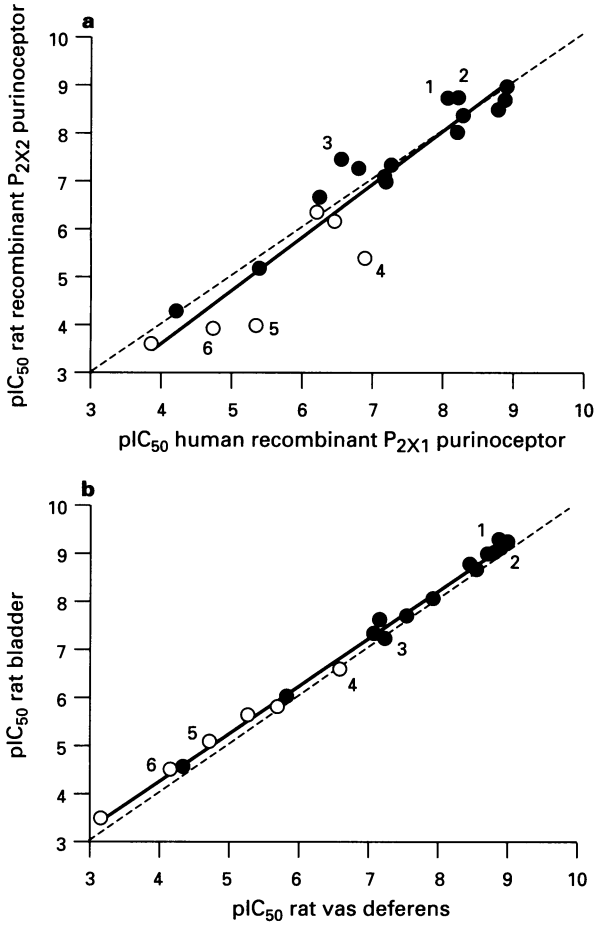

Figure 1 Comparison of P_{2X} purinoceptor subtypes labelled with [^{35}S]-ATPγS. (a) A comparison of affinity estimates for the human bladder recombinant P_{2X1} purinoceptor and rat PC12 cell recombinant P_{2X2} purinoceptor when expressed in CHO-K1 cells using Semliki forest virus (correlation coefficient $r = 0.894$). (b) A comparison of affinity estimates obtained at the [^{35}S]-ATPγS binding sites labelled in rat vas deferens and rat bladder membranes (correlation coefficient $r = 0.995$). In each case the data are for a series of nucleotides (●) and for five P_{2X} purinoceptor antagonists and dextran sulphate (○). The dotted line is the line of identity while the solid line represents the regression line for the entire data set. Only those compounds which exhibited a greater than 3 fold selectivity between the human recombinant P_{2X1} and rat recombinant P_{2X2} purinoceptors are indicated and are numbered as ; (1) ATPγS, (2) 2-meS-ATP, (3) 2'-deoxy ATP, (4) dextran sulphate, (5) DIDS, (6) PPADS.

Comparison of endogenous P_{2X1} purinoceptors in rat vas deferens and rat bladder membranes

The affinity estimates at the [^{35}S]-ATPγS binding sites present in rat vas deferens and rat bladder membranes were essentially the same for all compounds examined (Table 2; Figure 1b; $r = 0.995$) suggesting that there are no marked differences in the endogenous P_{2X1} purinoceptors in these two tissues.

Comparison of the rat recombinant P_{2X2} purinoceptor and endogenous P_{2X1} purinoceptors

The binding characteristics of the rat recombinant P_{2X2} purinoceptor were marginally different from those of the endogenous P_{2X1} purinoceptors present in rat vas deferens ($r = 0.915$) and rat bladder ($r = 0.932$) membranes (cf. Tables 1 and 2). In particular, $\beta\gamma$imATP, DIDS and dextran sulphate possessed 6 to 17 fold lower affinity for rat recombinant P_{2X2} purinoceptors than for rat endogenous P_{2X1} purinoceptors, while suramin and cibacron blue possessed approximately 3 to 8 fold higher affinity for the rat recombinant P_{2X2} purinoceptor than for rat endogenous P_{2X1} purinoceptors.

Comparison of [^{35}S]-ATPγS binding at human recombinant P_{2X1} purinoceptors and rat endogenous P_{2X1} purinoceptors

The affinity estimates obtained at the human recombinant P_{2X1} purinoceptor were slightly different from those obtained at the rat endogenous P_{2X1} purinoceptors (Figure 2). Of note, $\beta\gamma$meATP possessed 10 fold higher affinity for the rat endogenous P_{2X1} purinoceptors than for the human recombinant P_{2X1} purinoceptor, while suramin possessed 7 to 16 fold higher affinity for the human recombinant P_{2X1} purinoceptor than for the rat endogenous P_{2X1} purinoceptors.

Discussion

The main finding of this study is that the rat recombinant P_{2X2} purinoceptor can be labelled with high affinity using [^{35}S]-ATPγS, but not using [^{3}H]-$\alpha\beta$meATP. Furthermore, it is apparent that the two recombinant P_{2X} purinoceptor subtypes labelled with [^{35}S]-ATPγS, namely the human bladder P_{2X1} and rat PC12 cell P_{2X2} purinoceptors, display only relatively subtle differences in their binding characteristics.

CHO-K1 cells infected with the rat recombinant P_{2X2} purinoceptor using SFV possessed a much greater density of high affinity binding sites for [^{35}S]-ATPγS than were present in non-infected CHO-K1 cells. Furthermore, the characteristics of the sites in these 'rat recombinant P_{2X2} purinoceptor-infected' CHO-K1 cells were very different from those previously described in the non-infected CHO-K1 cells membranes (Michel *et al.*, 1996). The additional finding that functional P_{2X} purinoceptors were detected in electrophysiological studies on the 'rat recombinant P_{2X2} purinoceptor-infected' CHO-K1 cells, but not in non-infected or SFV-LacZ infected CHO-K1 cells, suggests that [^{35}S]-ATPγS was labelling the rat recombinant P_{2X2} purinoceptor expressed in these CHO-K1 cells.

The rat recombinant P_{2X2} purinoceptor displayed a number of differences from the human recombinant P_{2X1} purinoceptor. The biggest difference between the two recombinant receptors was in their ability to bind [^{3}H]-$\alpha\beta$meATP with high affinity in a calcium-dependent manner. We have previously shown that calcium ions produce a 63 fold increase in the affinity of [^{3}H]-$\alpha\beta$meATP for the endogenous P_{2X1} purinoceptor in rat vas

Table 2 Competition binding studies at the P_{2X} purinoceptors labelled with [^{35}S]-ATPγS in rat vas deferens and bladder membranes

Compound	Bladder		Vas deferens	
	pIC_{50}	Hill slope	pIC_{50}	Hill Slope
ATP	9.20±0.13	0.82±0.04	9.01±0.08	0.85±0.10
ATPγS	8.96±0.10	0.81±0.03	8.73±0.10	0.89±0.04
2-me-S-ATP	8.98±0.03	0.73±0.09	8.79±0.12	0.68±0.03
αβmeATP	7.68±0.08	0.83±0.05	7.57±0.14	0.92±0.06
ADP	7.59±0.04	0.95±0.12	7.18±0.13	0.96±0.13
βγmeATP	7.21±0.06	0.96±0.10	7.24±0.11	0.84±0.04
L-βγme ATP	6.00±0.10	0.64±0.10	5.83±0.09	0.88±0.06
αβmeADP	4.59±0.15	0.51±0.07	4.36±0.11	0.83±0.07
2-Cl-ATP	9.25±0.04	0.90±0.04	8.88±0.02	0.69±0.04
APPPP	8.73±0.03	0.87±0.10	8.48±0.04	0.91±0.05
ATPαS	9.09±0.17	0.89±0.05	8.90±0.01	0.91±0.06
AP5A	7.32±0.06	0.93±0.03	7.10±0.06	0.93±0.06
βγimATP	8.04±0.11	0.92±0.11	7.95±0.02	0.91±0.04
Dibenzyl-ATP	8.66±0.01	1.10±0.01	8.56±0.05	0.71±0.07
Deoxy-ATP	7.24±0.06	0.91±0.11	7.26±0.05	1.01±0.02
Dextran	6.60±0.09	0.68±0.02	6.60±0.07	0.79±0.07
DIDS	5.09±0.07	0.94±0.13	4.74±0.04	1.00±0.02
PPADS	4.51±0.04	0.83±0.03	4.18±0.22	0.98±0.28
PPO_4	3.47±0.07	0.72±0.08	3.18±0.08	0.69±0.02
Cib blue	5.81±0.07	1.40±0.20	5.71±0.03	1.39±0.03
Suramin	5.61±0.02	0.62±0.02	5.26±0.07	0.70±0.03

Competition binding studies on the sites labelled on 0.15 nM [^{35}S]ATPγS in membranes prepared from rat vas deferens or rat bladder. The data are the mean±s.e.mean of n = 3–5 determinations. The data for ATP, ATPγS, 2-meS-ATP, αβmeATP, ADP, βγmeATP, L-βγmeATP and αβmeADP at the rat vas deferens P_{2X1} purinoceptor are from Michel & Humphrey (1996).

deferens membranes (Michel & Humphrey, 1994). A similar phenomenon occurs at the endogenous P_{2X1} purinoceptor in rat bladder membranes (A.D. Michel, unpublished data) and at the human bladder recombinant P_{2X1} purinoceptor. Thus, in the presence of calcium ions [^{3}H]-αβmeATP bound with high affinity to the human recombinant P_{2X1} purinoceptor (pK_d for [^{3}H]-αβmeATP = 9.2; pIC_{50} for αβmeATP = 8.2) but in the absence of divalent cations, αβmeATP possessed relatively low affinity (pIC_{50} = 7.2) for the human recombinant P_{2X1} purinoceptor when labelled with [^{35}S]-ATPγS (Michel *et al.*, 1996).

In marked contrast, calcium-dependent, high affinity binding of [^{3}H]-αβmeATP to the rat recombinant P_{2X2} purinoceptor could not be detected, and it appeared that αβmeATP possessed only modest affinity (pIC_{50} = 6.8) for the [^{3}H]-αβmeATP binding sites in the 'rat recombinant P_{2X2} purinoceptor-infected' CHO-K1 cells. Indeed, due to the low affinity of the radioligand and the paucity of membrane material, it was not possible to study the [^{3}H]-αβmeATP binding sites in the 'rat recombinant P_{2X2} purinoceptor-infected' CHO-K1 cells in detail. It should be noted that the failure to detect high affinity binding of [^{3}H]-αβmeATP to the rat recombinant P_{2X2} purinoceptor was not due to low receptor expression. On the contrary, the level of receptor expression for the rat recombinant P_{2X2} purinoceptor in this study (B_{max} = 18.1 pmol mg^{-1} protein of [^{35}S]-ATPγS binding sites) was greater than that for the human recombinant P_{2X1} purinoceptor (B_{max} = 6.8 pmol mg^{-1} protein of [^{35}S]-ATPγS binding sites) where it has been possible to demonstrate high affinity binding of [^{3}H]-αβmeATP (Michel *et al.*, 1996).

Since αβmeATP possessed relatively low affinity for both the human recombinant P_{2X1} purinoceptor and rat recombinant P_{2X2} purinoceptor (pIC_{50} of 7.2 and 7.1, respectively) when labelled with [^{35}S]-ATPγS in the absence of calcium, but possessed high affinity for the human P_{2X1} purinoceptor when studied in the presence of calcium it would appear that a major difference between these two recombinant P_{2X} purinoceptors is in the calcium-sensitivity of αβmeATP binding. Whether, this effect of calcium is directly upon the receptor protein or is due to its binding to αβmeATP is not known at present but is worthy of further study. Indeed it is intriguing that αβmeATP can bind to the rat recombinant P_{2X2} purinoceptor labelled using [^{35}S]-ATPγS with an affinity of approximately 100 nM, yet it possesses no appreciable agonist

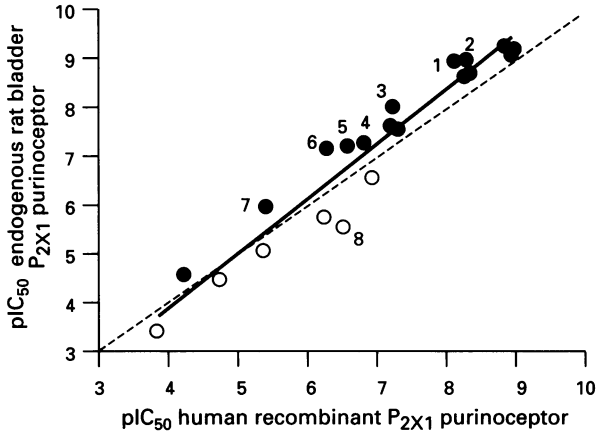

Figure 2 Comparison of the human recombinant and rat endogenous P_{2X1} purinoceptors. Affinity estimates for the human recombinant P_{2X1} purinoceptor, expressed in CHO-K1 cells using Semliki forest virus, are compared with those determined at the [^{35}S]-ATPγS binding sites present in rat bladder membranes. In each case the data are for a series of nucleotides (●) and for five P_{2X} purinoceptor antagonists and dextran sulphate (○). The dotted line is the line of identity while the solid line represents the regression line for the entire data set. The correlation coefficient was 0.932. Compounds which exhibited greater than 3 fold selectivity for either the human recombinant P_{2X1} purinoceptor or the endogenous rat bladder P_{2X1} purinoceptor are numbered as: (1) ATPγS, (2) 2-meS-ATP, (3) βγimATP, (4) AP5A, (5) 2'-deoxy ATP, (6) βγmeATP, (7) L-βγmeATP, (8) suramin.

or antagonist action at this receptor subtype in functional studies when studied at concentrations up to 100 μM (Evans *et al.*, 1995).

Surprisingly, the affinity estimates for the other nucleotides tested at the rat recombinant P_{2X2} and human recombinant P_{2X1} purinoceptor, determined using [^{35}S]-ATPγS, did not differ greatly with the possible exception of deoxy-ATP which possessed 8 fold higher affinity for the rat recombinant P_{2X2} than for the human recombinant P_{2X1} purinoceptor. Some differences were also observed in affinity estimates for the an-

tagonists, with PPADS and DIDS possessing 7 to 24 fold higher affinity for human recombinant P_{2X1} than for rat recombinant P_{2X2} purinoceptors. Although, these antagonists can be used to discriminate between the recombinant P_{2X} purinoceptors in binding studies, the relevance of these results to functional studies on the P_{2X} purinoceptor is not certain. Thus, previous binding studies using PPADS have suggested that it binds very slowly to P_{2X} purinoceptors, labelled with [^{3}H]-$\alpha\beta$meATP, and that this can complicate the extrapolation of binding data to functional studies (Khakh *et al.*, 1994). Similarly, DIDS appears to be a non-competitive, pseudo-irreversible P_{2X} purinoceptor antagonist (Bültmann & Starke, 1994) and this may complicate its use in functional receptor characterization. Nevertheless, a recent functional study using the recombinant P_{2X} purinoceptor subtypes has shown that DIDS can differentiate between P_{2X} purinoceptors and that it possesses higher affinity for human P_{2X1} than for rat P_{2X2} purinoceptors (Evans *et al.*, 1995).

Dextran sulphate was the most selective agent for discriminating between human P_{2X1} and rat P_{2X2} purinoceptors possessing 33 fold higher affinity for the human recombinant P_{2X1} than for the rat recombinant P_{2X2} purinoceptor. Previous studies have shown that dextran sulphate is also a very potent inhibitor of [^{3}H]-$\alpha\beta$meATP binding to the rat vas deferens P_{2X1} purinoceptor (Michel *et al.*, 1994) and in this study dextran sulphate also possessed high affinity for the endogenous P_{2X1} purinoceptors of rat vas deferens and rat bladder, labelled with [^{35}S]-ATPγS. Interestingly, dextran sulphate can also distinguish between the [^{3}H]-$\alpha\beta$meATP binding sites in rat vas deferens and rat brain (Michel *et al.*, 1994). It is unlikely, however, that dextran sulphate will be useful in functional studies since its affinity for the [^{35}S]-ATPγS binding sites in rat vas deferens is decreased up to 100 fold in the presence of a physiological concentration (140 mM) of sodium chloride (A.D. Michel, unpublished observation). This effect is similar to that described at the Ca-ATPase enzyme where it has been shown that monovalent cations decrease the potency of dextran sulphate as a Ca-ATPase inhibitor (De Meis & Suzano, 1994).

In the present study we have further characterized the [^{35}S]-ATPγS binding sites in rat vas deferens and also studied the [^{35}S]-ATPγS binding sites in rat bladder since the source of the human recombinant P_{2X1} purinoceptor was the bladder. The binding characteristics of the endogenous [^{35}S]-ATPγS binding sites in the rat vas deferens and rat bladder were almost identical ($r=0.995$) with respect to the 21 compounds examined suggesting that the [^{35}S]-ATPγS binding sites in these two rat tissues are the same. This is consistent with the studies of Valera *et al.* (1994), who have demonstrated high levels of mRNA for the vas deferens P_{2X1} purinoceptor in the rat bladder indicating that the bladder contains the same P_{2X1} purinoceptor as is present in the rat vas deferens.

We have also provided some evidence for a possible species difference between the smooth muscle P_{2X1} purinoceptors. Thus, although the endogenous and recombinant P_{2X1} purinoceptors could all bind [^{3}H]-$\alpha\beta$meATP with high affinity, there were differences in affinity estimates between the human recombinant P_{2X1} purinoceptor and the P_{2X1} purinoceptors in rat vas deferens and bladder membranes when these P_{2X1} purinoceptors were labelled with [^{35}S]-ATPγS. In particular, $\alpha\beta$meATP, $\beta\gamma$meATP, deoxyATP and $\beta\gamma$imATP possessed lower affinity at the human recombinant P_{2X1} purinoceptor than at the endogenous rat P_{2X1} purinoceptors while affinity estimates for the five antagonists were generally higher at the human recombinant P_{2X1} purinoceptor. These differences were reflected in the lower correlation coefficients obtained when comparing across species between the human recombinant P_{2X1} purinoceptors and the rat endogenous P_{2X1} purinoceptors ($r=0.915$ to 0.932), compared with the excellent correlation obtained when comparing between the endogenous P_{2X1} purinoceptors in rat vas deferens and rat bladder ($r=0.995$).

In summary, [^{35}S]-ATPγS can be used to label the rat recombinant P_{2X2} and human recombinant P_{2X1} purinoceptors. Despite the marked differences reported between the PC12 (P_{2X2}) and smooth muscle (P_{2X1}) forms of P_{2X} purinoceptor in functional studies, the differences between these P_{2X} purinoceptors in binding studies were more limited. However, a number of antagonists could discriminate between the P_{2X} purinoceptor subtypes in the binding studies raising expectations that selective antagonists for these receptors can be developed.

We would like to thank Daniele Estoppey for her expert help in culturing the CHO-K1 cells and performing the SFV infections.

References

ABBRACCHIO, M.P. & BURNSTOCK, G. (1994). Purinoceptors: Are there families of P2X and P2Y purinoceptors? *Pharmacol. Ther.*, **64**, 445–475.

BO, X., ZHANG, Y., NASSAR, M., BURNSTOCK, G. & SCHOEPFER, R. (1995). A P2X purinoceptor cDNA conferring a novel pharmacological profile. *FEBS Lett.*, **375**, 129–133.

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

BÜLTMANN, R. & STARKE, K. (1994). Blockade by 4,4'-diisothiocyanatostilbene-2,2'-disulphonate (DIDS) of P_{2X} purinoceptors in rat vas deferens. *Br. J. Pharmacol.*, **112**, 690–694.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

CHEN, C-C., AKOPIAN, A.N., SIVILOTTI, L., COLQUHOUN, D., BURNSTOCK, G. & WOOD, J.N. (1995). A P2X-purinoceptor expressed by a subset of sensory neurons. *Nature*, **377**, 428–431.

CHENG, Y. & PRUSSOF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of an inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099–3108.

DE MEIS, L. & SUZANO, V.A. (1994). Uncoupling of muscle and blood platelets Ca^{2+} transport ATPases by heparin. Regulation by K^+. *J. Biol. Chem.*, **269**, 14525–14529.

EVANS, R.J. & KENNEDY, C. (1994). Characterization of P_2-purinoceptors in the smooth muscle of the rat tail artery: a comparison between contractile and electrophysiological responses. *Br. J. Pharmacol.*, **113**, 853–860.

EVANS, R.J., LEWIS, C., BUELL, G., VALERA, S., NORTH, R.A. & SURPRENANT, A. (1995). Pharmacological characterisation of heterolologously expressed ATP-gated cation channels (P_{2X}-purinoceptors). *Mol. Pharmacol.*, **48**, 178–183.

FREDHOLM, B.B., ABBRACCHIO, M.P., BURNSTOCK, G., DALY, J.W., HARDEN, T.K., JACOBSON, K.A., LEFF, P. & WILLIAMS, M. (1994). Nomenclature and classification of purinoceptors. *Pharmacol. Rev.*, **46**, 143–156.

HUMPHREY, P.P.A., BUELL, G., KENNEDY, I., KHAKH, B.S., MICHEL, A.D., AURPRENANT, A. & TREZISE, D.J. (1995). New insights on P_{2X} purinoceptors. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **352**, 585–596.

INOUE, R. & BRADING, A.F. (1990). The properties of the ATP-induced depolarization and current in single cells isolated from the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **100**, 619–625.

KHAKH, B.S., HUMPHREY, P.P.A. & SURPRENANT, A. (1995a). Electrophysiological properties of P_{2X} purinoceptors in rat superior cervical, nodose and guinea-pig coeliac neurones. *J. Physiol.*, **484**, 385–395.

KHAKH, B.S., MICHEL, A. & HUMPHREY, P.P. (1994). Estimates of antagonist affinities at P_{2X} purinoceptors in rat vas deferens. *Eur. J. Pharmacol.*, **263,** 301–309.

KHAKH, B.S., SURPRENANT, A. & HUMPHREY, P.P.A. (1995b). A study of P_{2X} purinoceptors mediating the electrophysiological and contractile effects of purine nucleotides in rat vas deferens. *Br. J. Pharmacol.*, **115,** 177–185.

LEWIS, C., NEIDHART, S., HOLY, C., NORTH, R.A., BUELL, G. & SURPRENANT, A. (1995). Coexpression of P_{2X2} and P_{2X3} receptor can account for ATP-gated currents in sensory neurons. *Nature*, **377,** 432–435.

LILJESTRÖM, P. & GAROFF, H. (1991). A new generation of animal cell expression vectors based on the Semliki forest virus replicon. *Biotechnology*, **9,** 1356–1361.

LUNDSTRÖM, K., MILLS, A., BUELL, G., ALLET, E., ADAMI, N. & LILJESTRÖM, P. (1994). High-level expression of the human neurokinin-1 receptor in mammalian celllines using the Semliki Forest virus expression system. *Eur. J. Biochem.*, **224,** 917–921.

MICHEL, A.D. & HUMPHREY, P.P.A. (1993). Distribution and characterisation of [^{3}H]alpha-beta-methylene ATP binding sites in the rat. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **348,** 608–617.

MICHEL, A.D. & HUMPHREY, P.P.A. (1994). Effects of metal cations on [^{3}H]alpha,beta-methylene ATP binding in rat vas deferens. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **350,** 113–122.

MICHEL, A.D. & HUMPHREY, P.P.A. (1996). High affinity P_{2X} purinoceptor binding sites for [^{35}S]adenosine 5'-O-[3-thiotriphosphate] in rat vas deferens membranes. *Br. J. Pharmacol.*, **117,** 63–70.

MICHEL, A.D., LUNDSTROM, K., BUELL, G.N., SURPRENANT, A., VALERA, S. & HUMPHREY, P.P.A. (1996). The binding characteristics of a human bladder recombinant P_{2X} purinoceptor, labelled using [^{3}H]$\alpha\beta$meATP, [^{35}S]ATPγS or [^{33}P]ATP. *Br. J. Pharmacol.*, **117,** 1254–1260.

MICHEL, A.D., TREZISE, D.J., GRAHAMES, C.B.A., KHAKH, B.S. & HUMPHREY, P.P.A. (1994). The selective P_{2X} purinoceptor agonist, L-β,γ-methylene ATP, discriminates between smooth muscle and neuronal P_{2X} purinoceptors. *Br. J. Pharmacol.*, **113,** 58P.

TREZISE, D.J., MICHEL, A.D., GRAHAMES, C.B.A., KHAKH, B.S., SURPRENANT, A. & HUMPHREY, P.P.A. (1995). The selective P2x purinoceptor agonist, beta, gamma methylene L-ATP, discriminates between smooth muscle and neuronal P_{2X} purinoceptors. *Naunyn Schmiedeberg's Arch. Pharmacol.*, **351,** 603–609.

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

VALERA, S., TALABOT, F., EVANS, R.J., GOS, A., ANTINARAKIS, S.E., MORRIS, M.A. & BUELL, G. (1995). Characterisation and chromosomal localisation of a human P_{2X} receptor from urinary bladder. *Recept. Channels*, **3,** 283–289.

(Received January 22, 1996
Revised March 29, 1996
Accepted April 15, 1996)