British Journal of Pharmacology (1996) 117, 1254–1260

The binding characteristics of a human bladder recombinant P_{2x} purinoceptor, labelled with [^{3}H]-$\alpha\beta$meATP, [^{35}S]-ATPγS or [^{33}P]-ATP

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1 The binding of [^{3}H]-$\alpha\beta$meATP, [^{35}S]-ATPγS and [α^{33}P]-ATP to a human bladder P_{2X} purinoceptor, transiently expressed in CHO-K1 cells using the Semliki Forest Virus (SFV) expression system, was examined. The characteristics of the binding sites were compared with results obtained in rat vas deferens, a tissue in which the radioligands are thought to label P_{2X} purinoceptors and in which the endogenous P_{2X} purinoceptor displays high homology with the human bladder P_{2X} purinoceptor.

2 In non-infected CHO-K1 cells, 100 μM ATP evoked only small inward currents (40 pA) in approximately 30% of the cells when studied by the whole-cell voltage clamp technique. In membranes prepared from either these non-infected cells or cells infected with SFV containing the LacZ gene (SFV-LacZ), [^{3}H]-$\alpha\beta$meATP bound with low affinity ($pK_d = 7.04$; $B_{max} = 8.88$ pmol ml^{-1} protein) and there was only a low density of [^{35}S]-ATPγS binding sites ($pK_d = 8.74$; $B_{max} = 358$ fmol ml^{-1} protein). These binding sites differed from those present in rat vas deferens. Thus, pIC_{50} values for $\alpha\beta$meATP (6.5) and L-$\beta\gamma$meATP (4.0) at the [^{3}H]-$\alpha\beta$meATP binding sites in non-infected CHO-K1 cells were much lower than the respective pIC_{50} values of 8.3 and 7.7, determined in rat vas deferens. Similarly, affinity estimates (pIC_{50} values) for ATP (6.82), 2-meS-ATP (5.43), ATPγS (7.06) and $\alpha\beta$meATP (4.84) at the [^{35}S]-ATPγS binding sites in non-infected CHO-K1 cells were up to 2291 fold lower than the respective values of 9.01, 8.79, 8.73 and 7.57, determined in rat vas deferens.

3 In CHO-K1 cells infected using SFV containing the cDNA for the human bladder P_{2X} purinoceptor (SFV-h.P_{2X}), ATP, 2-meS-ATP and $\alpha\beta$meATP evoked large inward currents (2–7 nA) in whole cell voltage clamp studies. In membranes prepared from these SFV-h.P_{2X} infected cells, [^{3}H]-$\alpha\beta$meATP binding was increased, compared to that measured in the non infected or SFV-LacZ infected cells, with only high affinity [^{3}H]-$\alpha\beta$meATP binding sites being detected ($pK_d = 9.21$; $B_{max} = 3.54$ pmol mg^{-1} protein). The pIC_{50} values for $\alpha\beta$meATP (8.2) and L-$\beta\gamma$meATP (7.2) in competing for these sites were the same or similar to the values determined in rat vas deferens.

4 A high density of [^{35}S]-ATPγS binding sites ($pK_d = 9.09$; $B_{max} = 6.82$ pmol mg^{-1} protein) was also present in the membranes from CHO-K1 cells infected with SFV-h.P_{2X} and affinity estimates (pIC_{50} values) for ATP (8.93), 2-meS-ATP (8.23), ATPγS (8.08), and $\alpha\beta$meATP (7.17) at competing for these sites were as much as 631 fold higher than the respective values determined in non-infected CHO-K1 cells but were close to the values determined in rat vas deferens. Similar data were obtained with [α^{33}P]-ATP as radioligand.

5 These data suggest that [^{3}H]-$\alpha\beta$meATP, [^{35}S]-ATPγS and [^{33}P]-ATP label the human bladder recombinant P_{2X} purinoceptor expressed in CHO-K1 cells following infection with SFV-h.P_{2X} and provide further corroborative evidence to support the contention that the high affinity binding sites for these radioligands in rat vas deferens are P_{2X} purinoceptors.

Keywords: [^{3}H]-$\alpha\beta$meATP; [^{35}S]-ATPγS; [α^{33}P]-ATP; rat vas deferens; human bladder P_{2X} purinoceptor; Semliki forest virus; CHO-K1 cells

Introduction

The extracellular receptors for ATP, the so-called P_2 purinoceptors, exist as at least 5 types denoted as P_{2X}, P_{2y}, P_{2z}, P_{2t} and P_{2u} (Fredholm *et al.*, 1994). The P_{2X} purinoceptor has been characterized extensively in functional studies and on the basis of both pharmacological and electrophysiological studies has been shown to be a ligand-gated cation channel (Bean, 1992). Indeed it has been suggested that all ATP-gated cation channels should be in a class called P_{2X}, with further division into subtypes (Abbracchio & Burnstock, 1994).

Despite extensive functional studies P_{2X} purinoceptors have not been examined in detail at the ligand binding level. This has been due to the lack of suitable high affinity antagonists of the P_{2X} purinoceptor and to the metabolic instability of radiolabelled purine nucleotides. Recently, however, the metabolically stable P_{2X} purinoceptor agonist radioligand, [^{3}H]-α,β-methylene ATP ([^{3}H]-$\alpha\beta$meATP) has been used in binding studies (Bo & Burnstock, 1990; Bo *et al.*, 1992). This radioligand labels high affinity sites in a number of tissues including the rat vas deferens and rat bladder, two tissues which are known to express the P_{2X} purinoceptor from functional studies (Burnstock & Kennedy, 1985). Since the rank order of binding affinities at these sites of α,β-methylene ATP ($\alpha\beta$meATP) > 2-methylthio-ATP (2-meS-ATP) = ATP was similar to the rank order of agonist potencies expected for a P_{2X}

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purinoceptor it has been proposed that these sites are P_{2X} purinoceptors (Bo *et al.*, 1992; Michel & Humphrey, 1993). In addition, the finding that affinity estimates for several P_{2X} purinoceptor antagonists at the [^{3}H]-$\alpha\beta$meATP binding sites are similar to estimates of their affinity determined in functional studies on the P_{2X} purinoceptors, has provided further corroborative evidence that the [^{3}H]-$\alpha\beta$meATP binding sites are P_{2X} purinoceptors (Bo *et al.*, 1992; Khakh *et al.*, 1994).

However, definitive proof that the [^{3}H]-$\alpha\beta$meATP binding sites correspond to P_{2X} purinoceptors has been difficult to provide for a number of reasons. First, the selectivity and specificity of the available P_{2X} purinoceptor antagonists is clearly limited as indicated by the multiplicity of actions of suramin (Voogd *et al.*, 1993). Furthermore, even the use of rank orders of agonist potency, the most widely accepted method for classifying the P_{2X} purinoceptor, is now being called into question. Thus, in functional studies it is apparent that the rank order of agonist potency of $\alpha\beta$meATP > 2-meS-ATP = ATP at the vas deferens P_{2X} purinoceptor (Burnstock & Kennedy, 1985) may be artifactual, resulting from the differential metabolism of 2-meS-ATP and ATP and instead the true rank order of potency is 2-meS-ATP = ATP $\geqslant \alpha\beta$meATP (Trezise *et al.*, 1994; Valera *et al.*, 1994; Evans & Kennedy, 1994; Khakh *et al.*, 1995a,b).

Secondly, the binding characteristics of [^{3}H]-$\alpha\beta$meATP have proved to be complex. Thus, recent evidence suggests that the high affinity [^{3}H]-$\alpha\beta$meATP binding sites in rat brain and rat vas deferens differ, possibly reflecting the presence of P_{2X} purinoceptor subtypes (Michel *et al.*, 1994), while high affinity binding of [^{3}H]-$\alpha\beta$meATP to a 5'-nucleotidase has been demonstrated in an endothelial-derived cell line (Michel *et al.*, 1995). In addition, low affinity sites for [^{3}H]-$\alpha\beta$meATP are present in all tissues studied and these sites, which presumably represent labelling of membrane bound ATPases and ATP-dependent proteins, can complicate the study of the high affinity binding sites for [^{3}H]-$\alpha\beta$meATP in those tissues possessing an appreciable proportion of the low affinity sites (Michel & Humphrey, 1993).

Recently, the cDNA sequences encoding two distinct P_{2X} purinoceptors have been cloned from rat vas deferens (Valera *et al.*, 1994) and the PC12 cell line (Brake *et al.*, 1994) and, when expressed in *xenopus* oocytes, the products of these genes reconstitute the properties of the endogenous P_{2X} purinoceptors present in rat vas deferens and PC12 cells, respectively. Given, the lack of suitable pharmacological tools and the potential problems of defining the rank order of agonist potency at the P_{2X} purinoceptor in tissues, the availability of recombinant P_{2X} purinoceptors in high density should provide a more definitive method for determining whether [^{3}H]-$\alpha\beta$meATP can indeed label the P_{2X} purinoceptor. To this end, we have determined if the recombinant P_{2X} purinoceptor, expressed in CHO-K1 cells, can be directly labelled. For these studies, the human bladder P_{2X} purinoceptor (Valera *et al.*, 1995), which displays high homology with the P_{2X} purinoceptor cloned from rat vas deferens (Valera *et al.*, 1994), was expressed in CHO-K1 cells with the aid of the Semliki Forest virus (SFV) expression system (Liljeström & Garoff, 1991; Lundström *et al.*, 1994) and the binding of [^{3}H]-$\alpha\beta$meATP to this recombinant receptor studied. In addition, binding studies were also performed with [^{35}S]-adenosine 5'-O-[3-thiotriphosphate] ([^{35}S]-ATPγS) and [α^{33}P]-ATP since previous reports have suggested that, in the absence of divalent cations, [^{35}S]-ATPγS can also specifically label the P_{2X} purinoceptor (Michel & Humphrey, 1996).

Methods

Expression of the human bladder P_{2X} purinoceptor in CHO-K1 cells

The human bladder P_{2X} purinoceptor coding region was amplified by PCR and cloned into the BamH1 site of the pSFV1 vector (Lundström *et al.*, 1994). *In vitro* transcripts of this plasmid 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 stock was collected 24 h later. CHO-K1 cells were grown as a monolayer culture in a 1:1 mixture of Dulbecco's modified Eagle's medium and Hams F12 essential amino acid mixture containing 10% foetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The CHO-K1 cells were infected with SFV containing the human bladder P_{2X} purinoceptor (SFV-h.P_{2X}) at a multiplicity of infection of 10 and were harvested 16 h later by incubation in divalent-cation free PBS. The data presented are from 3 separate infections. 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 human bladder P_{2X} purinoceptor coding region (SFV-lacZ).

Electrophysiological recordings

Conventional whole-cell recordings were obtained using an Axopatch 2D amplifier (Axon Instruments) with patch pipettes of 4–7 MΩ. Internal solution was (mM): K-aspartate 140, NaCl 20, EGTA 11 and HEPES 10 while the external solution was (mM): NaCl 140, KCl 2, $CaCl_2$ 2, $MgCl_2$ 1, glucose 12 and HEPES 5. The internal and external solutions were adjusted to pH 7.3 and the osmolarity was maintained at 305 mOsm. All studies were performed at room temperature and recordings were made 18–24 h after SFV infection of CHO-K1 cells. Cumulative concentration-response curves were constructed with agonists applied at intervals of 8–10 min.

Receptor binding studies

Vas deferens membranes were prepared from 200–350 g, CD derived, Sprague Dawley rats as described previously (Michel & Humphrey, 1993). CHO-K1 cells were homogenized in ice-cold 50 mM Tris, 1 mM EDTA assay buffer (pH 7.4 at 4°C) using a Polytron P10 tissue disrupter (2 × 10 s bursts on full setting). The cell homogenate was centrifuged at 48,000 *g* for 20 min and the crude membrane pellet resuspended in the same volume of assay buffer, supplemented with 4 mM $CaCl_2$. After a further centrifugation at 48,000 *g* for 20 min the pellet was resuspended in assay buffer and stored at −80°C until required. Receptor binding studies were performed as described previously for the study of [^{3}H]-$\alpha\beta$meATP binding to rat vas deferens (Michel & Humphrey, 1994) with a few minor modifications. When the binding of [^{35}S]-ATPγS or [α^{33}P]-ATP were examined, the 50 mM Tris, 1 mM EDTA assay buffer (pH 7.4 at 4°C) was used since [^{35}S]-ATPγS can only be used to label the P_{2X} purinoceptor in the absence of divalent cations (Michel & Humphrey, 1996). In the binding studies with [^{3}H]-$\alpha\beta$meATP, 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 either 3 h, in studies using [^{35}S]-ATPγS and [α^{33}P]-ATP, or 40 min when [^{3}H]-$\alpha\beta$meATP was the radioligand. Reactions were terminated by vacuum filtration over wet, 20 mM $Na_4P_2O_7$ pretreated, GF/B glass fibre filters using a cell harvester. The filters were washed for 10 s with 10 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.4 at 22°C) and bound radioligand was determined by liquid scintillation spectrophotometry using either a Packard Topcount or a Packard 2200CA scintillation counter.

In competition studies, the ability of a series of nucleotide analogues to compete for the binding sites labelled by 0.15 nM [^{35}S]-ATPγS, 0.1 nM [α^{33}P]-ATP or 1 nM [^{3}H]-$\alpha\beta$meATP was determined over a range of concentrations spanning at least 5 log units as described previously (Michel & Humphrey, 1994). In these studies, the non-specific binding (NSB) of [^{35}S]-ATPγS and [α^{33}P]-ATP was defined by use of 10 μM ATPγS while NSB of [^{3}H]-$\alpha\beta$meATP was defined by use of 30 μM $\alpha\beta$meATP.

In saturation studies, total binding and NSB of either 0.04–

6 nM [^{35}S]-ATPγS, 0.01–3 nM [α^{33}P]-ATP or 0.15–70 nM [^{3}H]-αβmeATP were measured. In these studies NSB of [^{3}H]-αβ meATP was defined using 10 μM L-β,γ-methylene ATP (L-βγmeATP) while NSB of [^{35}S]-ATPγS and [α^{33}P]-ATP was defined using 300 nM 2-meS-ATP. These concentrations of L-βγmeATP and 2-meS-ATP selectively inhibited high affinity binding of the radioligands to rat vas deferens membranes but produced minimal inhibition of binding to the low affinity sites present in the control CHO-K1 cell membranes (see Results) and were selected to enable only the high affinity binding sites for the radioligands to be quantified.

Data analysis

Saturation and competition binding data were analysed as described previously using iterative curve fitting procedures (Michel & Humphrey, 1994). The IC_{50} values that were determined were not adjusted to take account of the presence of radioligand, and are presented as the negative logarithm of the IC_{50} (pIC_{50}). Unless otherwise stated the data are the mean ± s.e.mean of 3–5 separate experiments.

Materials

The tetrasodium salt of 2-meS-ATP and sodium salt of L-βγmeATP were obtained from Research Biochemicals while the tetralithium salt of ATPγS, lithium salt of αβmeATP, sodium salt of α,β-methylene ADP (αβmeADP), sodium salt of ADP, disodium salt of ATP and sodium salt of βγmeATP were purchased from Sigma. [^{35}S]-ATPγS (specific activity 1500 Ci mmol^{-1}), [^{3}H]-αβmeATP (specific activity 28 Ci mmol^{-1}) and [α^{33}P]-ATP (specific activity 931–3000 Ci mmol^{-1}) were obtained from Amersham, UK.

Results

Functional and binding studies in non infected CHO-K1 cells

The CHO-K1 cell line was examined in both functional and binding studies to determine if it contained either functional P_{2X} purinoceptors or high affinity binding sites for [^{3}H]-αβ meATP and [^{35}S]-ATPγS.

In whole-cell voltage clamp studies, ATP (30–100 μM) evoked very small inward currents in about 30% (4 of 14) of the non infected CHO-K1 cells examined; the mean current to 100 μM ATP was 40 ± 8 pA (Figure 1a). In these cells only low affinity binding of [^{3}H]-αβmeATP binding was detected. Thus, in competition studies, specific [^{3}H]-αβmeATP binding, as defined by use of 30 μM αβmeATP, was inhibited with low affinity by αβmeATP (pIC_{50} = 6.51 ± 0.2, n_H = 0.74 ± 0.05) and by L-βγmeATP (pIC_{50} = 4.04 ± 0.32, n_H = 0.91 ± 0.05). In contrast, in rat vas deferens membranes αβmeATP (pIC_{50} = 8.25 ± 0.16, n_H = 0.85 ± 0.03) and L-βγmeATP (pIC_{50} = 7.70 ± 0.10) possessed higher affinity. When specific binding of [^{3}H]-αβmeATP was defined by use of 10 μM L-βγmeATP, the pK_d for [^{3}H]-αβmeATP binding to non infected CHO-K1 cell membranes, determined in saturation studies, was 7.04 ± 0.40 and the B_{max} 8.88 ± 4.93 pmol mg^{-1} protein, while in rat vas deferens membranes the pK_d was 9.41 ± 0.07 and the B_{max} 6.78 ± 2.8 pmol mg^{-1} protein (Figure 2a).

There were only low levels of [^{35}S]-ATPγS binding to non infected CHO-K1 cell membranes. In competition studies total binding of [^{35}S]-ATPγS to these membranes was inhibited by a number of nucleotide analogues, the most potent being ATP and ATPγS which inhibited binding maximally to the same extent (Figure 3a). The Hill slopes of the competition curves were less than unity for most compounds studied and affinity estimates were up to 2291 fold lower in the non infected CHO-K1 cells than in the vas deferens membranes (Table 1; Figure 4). In saturation studies on membranes from non infected CHO-K1 cells there were only low levels of high affinity specific [^{35}S]-ATPγS binding, as defined by use of 300 nM 2-meS-ATP, and although the data could be analysed assuming binding to a single population of sites (pK_d = 8.74 ± 0.06; B_{max} = 358 ± 137 fmol mg^{-1} protein), the accuracy of these estimates was limited. Thus, there was evidence for curvature in the Scatchard plot, suggestive of binding to more than one population of sites (Figure 2b) and specific binding represented only 8–17% of total binding. In contrast, in rat vas deferens membranes, [^{35}S]-ATPγS appeared to bind to a single population of high affinity sites with a pK_d of 9.41 ± 0.11 and B_{max} of 4.01 ± 0.52 pmol mg^{-1} protein (Figure 2c).

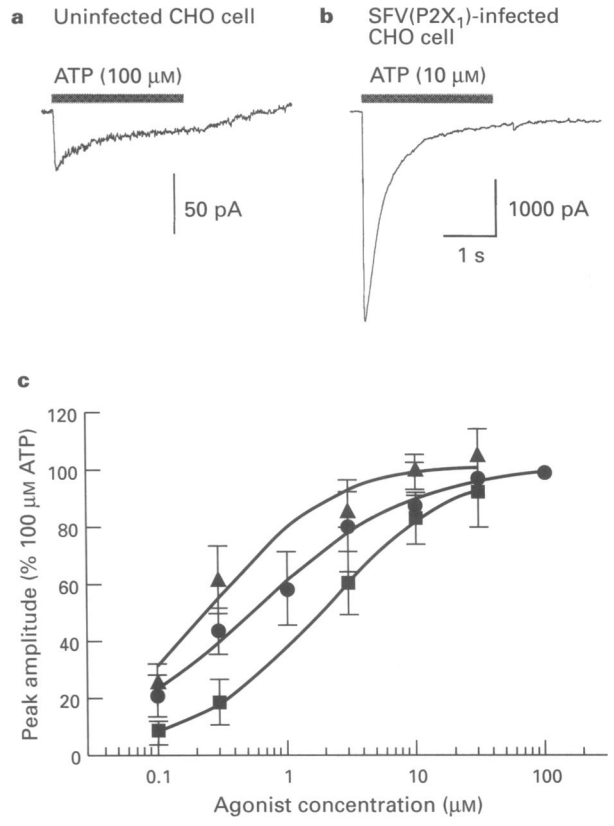

Figure 1 ATP-evoked currents in non infected CHO-K1 cells (a) and CHO-K1 cells infected with the human bladder P_{2X} purinoceptor using Semliki Forest virus (b,c). (a,b) Currents evoked in response to 2 s duration application of ATP (duration indicated by bars above traces) in non infected CHO-K1 cells and CHO-K1 cells infected with the human bladder P_{2X} purinoceptor using Semliki Forest virus (SFV-h.P_{2X}). Only 30% of non infected cells responded to ATP with inward currents while more than 95% of the SFV-h.P_{2X} infected cells were ATP-responsive (see text). (c) Concentration-response curves for inward currents to ATP (●), 2-meS-ATP (■) and αβmeATP (▲) in SFV-h.P_{2X} infected CHO-K1 cells. Each point is the mean ± s.e.mean of 3–8 cells. All recordings were obtained at a holding potential of −70 mV.

Binding and functional studies on the human bladder P_{2X} purinoceptor expressed in CHO-K1 cells

In approximately 95% of SFV-h.P_{2X} infected CHO-K1 cells, ATP (10 μM) evoked large currents ranging from 2000–7000 pA (4800 ± 450 pA; n = 15) (Figure 1b). Concentration-response curves to ATP, 2-meS-ATP and αβmeATP (Figure 1c) were similar to those previously obtained in HEK293 cells which had been transiently transfected with the cDNA encoding a vas deferens P_{2X} purinoceptor (P_{2X1}; Evans *et al.*, 1995). In addition, high affinity binding of [^{3}H]-αβmeATP could be detected in membranes prepared from these SFV-h.P_{2X} infected CHO-K1 cells. In competition experiments, αβ meATP and L-βγmeATP inhibited this [^{3}H]-αβmeATP binding to the same extent with pIC_{50} values of 8.21 ± 0.07 (n = 3) and

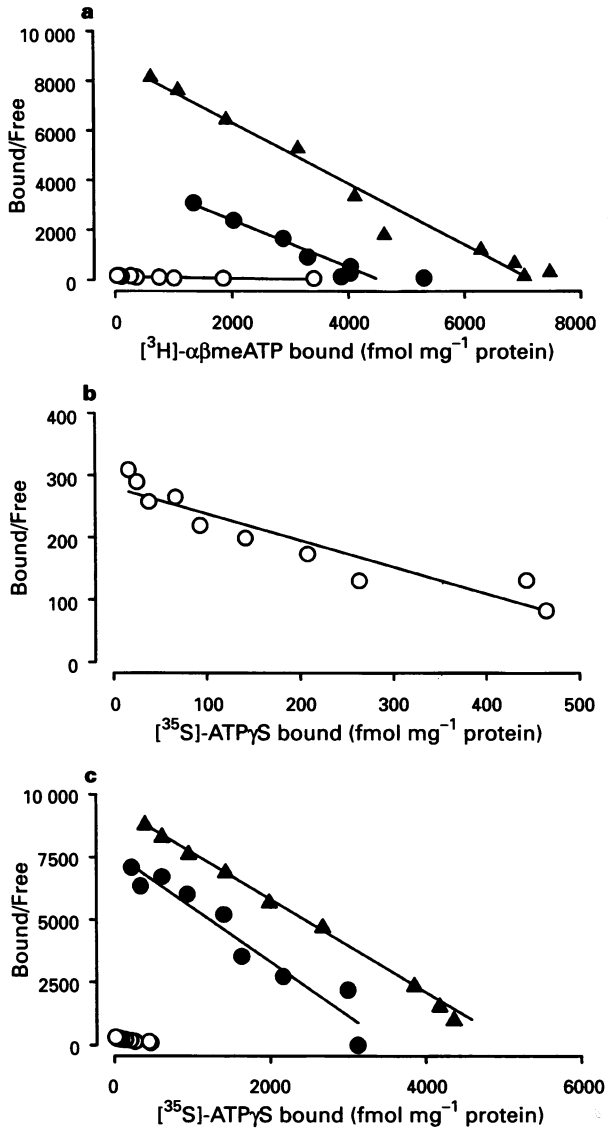

Figure 2 Saturation studies on the [^{3}H]-$\alpha\beta$meATP and [^{35}S]-ATPγS binding sites present in membranes prepared from non infected CHO-K1 cells, CHO-K1 cells infected with the human bladder P_{2X} purinoceptor using Semliki Forest virus (SFV-h.P_{2X}) or rat vas deferens. The data are Scatchard plots of the specific binding of [^{3}H]-$\alpha\beta$meATP (a) or [^{35}S]-ATPγS (b and c) to membranes prepared from (○) non infected CHO-K1 cells, (●) SFV-h.P_{2X} infected CHO-K1 cells or (▲) rat vas deferens. The data are from a single experiment in each case. The units of bound/free are fmol mg^{-1} protein nM^{-1}. In studies with [^{3}H]-$\alpha\beta$meATP non specific binding was defined using 10 μM L-$\beta\gamma$meATP while in studies using [^{35}S]-ATPγS non specific binding was defined by use of 300 nM 2-meS-ATP.

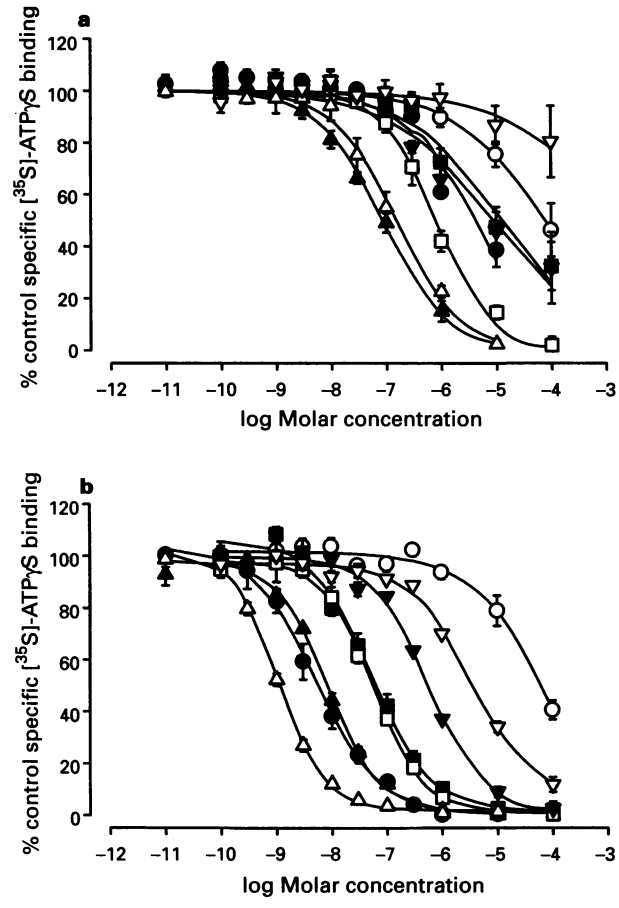

Figure 3 Competition studies on the [^{35}S]-ATPγS binding sites present in membranes from non infected CHO-K1 cells and CHO-K1 cells infected with the human bladder P_{2X} purinoceptor using the Semliki Forest virus (SFV-h.P_{2X}). The data are the mean ± s.e.mean of 3–4 experiments. Competition curves for ATP (△), 2-meS-ATP (●), ATPγS (▲), ADP (□), $\alpha\beta$meATP (■), $\beta\gamma$meATP, (▼). L-$\beta\gamma$meATP (▽) and $\alpha\beta$meADP (○) were obtained in membranes prepared from either (a) non infected CHO-K1 cells or (b) SFV-h.P_{2X} infected CHO-K1 cells. The radioligand concentration was approximately 0.15 nM and non specific binding (NSB) was defined by use of 10 μM ATPγS. Total and NSB levels in the non infected and CHO-K1 cells were approximately 2390 ± 630 and 318 ± 133 d.p.m. respectively, while in the SFV-h.P_{2X} infected CHO-K1 cells total and NSB were approximately 1846 ± 418 and 61 ± 12 d.p.m., respectively.

7.24 ± 0.14 (n = 3), respectively, while $\alpha\beta$meADP possessed only low affinity (pIC_{50} = 4.32 ± 0.21). In saturation studies, in which non specific binding was defined by use of 10 μM L-$\beta\gamma$meATP, [^{3}H]-$\alpha\beta$meATP bound to an apparently homogeneous population of high affinity sites in SFV-h.P_{2X} infected CHO-K1 cells with a pK_d of 9.21 ± 0.09 and B_{max} of 3.54 ± 1.11 pmol mg^{-1} protein (Figure 2a). When CHO-K1 cells were infected with SFV-lacZ there was no significant change in [^{3}H]-$\alpha\beta$meATP binding when compared to non infected cells (data not shown).

[^{35}S]-ATPγS binding was increased in membranes prepared from SFV-h.P_{2X} infected CHO-K1 cells when compared to binding measured in non infected cells. Thus, in saturation studies, in which non specific binding was defined by use of 300 nM 2-meS-ATP, [^{35}S]-ATPγS bound with high affinity to a single population of sites in membranes prepared from the SFV-h.P_{2X} infected CHO-K1 cells (pK_d = 9.09 ± 0.10; B_{max} = 6.82 ± 1.39 pmol mg^{-1} protein; Figure 2c). In competition studies, affinity estimates for a series of nucleotides at the [^{35}S]-ATPγS binding sites in the non infected and SFV-h.P_{2X} infected CHO-K1 cells differed by up to 631 fold (Figures 3 and 4 and Table 1). In contrast affinity estimates at the [^{35}S]-ATPγS binding sites in the SFV-h.P_{2X} infected CHO-K1 cells and the vas deferens were closely correlated (r = 0.95), differing by at most ten fold in the case of $\beta\gamma$meATP (Table 1; Figures 3 and 4). When CHO-K1 cells were infected with SFV-lacZ there was no significant change in [^{35}S]-ATPγS binding when compared to non infected cells (data not shown).

Radiolabelling studies using [α^{33}P]-ATP

In membranes prepared either from the vas deferens or the SFV-h.P_{2X} infected CHO-K1 cells, ATP possessed higher affinity than ATPγS (Table 1). A number of experiments were therefore performed using [α^{33}P]-ATP to determine if this radioligand could label the same sites as [^{35}S]-ATPγS. In saturation studies, in which non specific binding was defined with 300 nM 2-meS-ATP, [α^{33}P]-ATP bound to a single population

of sites in rat vas deferens membranes ($pK_d = 10.10 \pm 0.08$; $B_{max} = 2.30 \pm 0.15$ pmol mg^{-1} protein). In non infected CHO-K1 cells saturation binding of [α^{33}P]-ATP could be analysed assuming binding to a single population of sites ($pK_d = 9.49 \pm 0.21$; $B_{max} = 173 \pm 47$ fmol mg^{-1} protein; $n = 3$) although specific binding represented only 10–15% of total binding and the Scatchard plot showed marked signs of curvature suggesting heterogeneity of binding (data not shown). The affinity estimates of the nucleotides for the [α^{33}P]-ATP binding sites in the rat vas deferens membranes were almost identical to those determined with [^{35}S]-ATPγS (cf. Table 1 and 2; $r = 0.997$). In the non infected CHO-K1 cells the affinity estimates obtained with the two radioligands were not so closely correlated ($r = 0.811$), although the Hill slopes of competition curves using both radioligands were less than unity in these membranes. Following infection with SFV-h.P_{2X}, high affinity specific binding of [α^{33}P]-ATP to CHO-K1 cells was markedly increased when compared to binding measured in either non infected or SFV-lacZ infected CHO-K1 cells. Thus, in saturation studies, in which non specific binding was defined by use of 300 nM 2-meS-ATP, [α^{33}P]-ATP bound with high affinity to a single population of sites ($pK_d = 9.89 \pm 0.08$; $B_{max} = 15.4 \pm 1.7$ pmol mg^{-1} protein) in membranes from SFV-h.P_{2X} infected CHO-K1 cells. Affinity estimates determined at these sites in competition studies were almost identical to those determined with [^{35}S]-ATPγS as the radioligand (cf. Tables 1 and 2; $r = 0.993$). It should be noted that the studies with [α^{33}P]-ATP were performed on a different batch of membranes from those used in studies with [^{3}H]-$\alpha\beta$meATP and [^{35}S]-ATPγS and this may account for the higher B_{max} determined for [α^{33}P]-ATP.

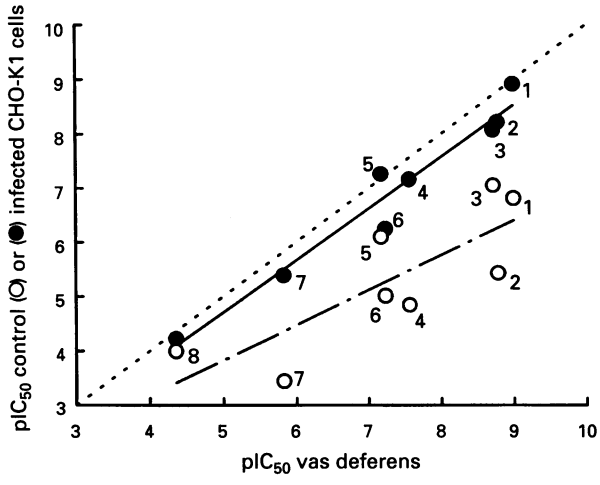

Figure 4 Comparison of [^{35}S]-ATPγS binding sites in rat vas deferens with the sites labelled in non infected CHO-K1 cells or CHO-K1 cells infected with human bladder P_{2X} purinoceptor using Semliki Forest virus (SFV-h.P_{2X}). Affinity estimates of nucleotides at the [^{35}S]-ATPγS binding sites of rat vas deferens membranes are compared with affinity estimates for the [^{35}S]-ATPγS binding sites present in membranes from either (○) non infected CHO-K1 cells or (●) SFV-h.P_{2X} infected CHO-K1 cells. The dotted line is the line of identity while the solid line represents the regression line obtained when comparing pIC_{50} values in rat vas deferens and the SFV-h.P_{2X} infected CHO-K1 cells ($r = 0.953$). The dashed line is the regression line for the comparison of pIC_{50} values in rat vas deferens and the non infected CHO-K1 cells ($r = 0.655$). The nucleotides were: (1) ATP, (2) 2-meS-ATP, (3) ATPγS, (4) $\alpha\beta$meATP, (5) ADP, (6) $\beta\gamma$meATP, (7) L-$\beta\gamma$meATP, (8) $\alpha\beta$meADP.

Discussion

The main finding of this study was that, following infection of CHO-K1 cells with SFV-h.P_{2X}, high affinity specific binding sites for [^{3}H]-$\alpha\beta$meATP, [^{35}S]-ATPγS and [α^{33}P]-ATP could be detected. Since the binding characteristics of these sites differed markedly from those present in non infected CHO-K1 cells this would suggest that these radioligands were labelling the human bladder recombinant P_{2X} purinoceptor. Furthermore, the binding characteristics of the sites labelled by the radioligands were almost identical to those determined in rat vas deferens membranes, suggesting that the high affinity binding sites for [^{3}H]-$\alpha\beta$meATP and [^{35}S]-ATPγS, which have previously been identified in the rat vas deferens (Bo *et al.*, 1992; Michel & Humphrey, 1993; 1996), are indeed P_{2X} purinoceptors.

Ideally in such studies the host cell used should not contain binding sites for the radioligand. However, in the present study this was not possible since all cell lines examined (rat-1, LTK, COS-7, BHK and HEK293), including the CHO-K1 cell line, contain some nucleotide displaceable binding. Nevertheless, the presence of these sites was not an insurmountable problem since the sites were present at a relatively low density and/or their binding characteristics were very different from the binding sites labelled by these radioligands in rat vas deferens membranes, suggesting that they do not represent labelling of an endogenous P_{2X} purinoceptor. Furthermore, previous studies on the CHO-K1 cell line have failed to identify P_{2X} purinoceptor-mediated responses (Iredale & Hill, 1993) and our functional studies, demonstrating that ATP evokes only a very small inward current in a small population of cells, also suggest that CHO-K1 cells do not possess clearly defined P_{2X} purinoceptors. Thus it is unlikely that the [^{3}H]-$\alpha\beta$meATP and [^{35}S]-ATPγS binding sites in the non infected CHO-K1 cells are P_{2X} purinoceptors and instead may represent labelling of ATPases or other ATP-dependent enzymes.

Table 1 Competition studies at the [^{35}S]-ATPγS binding sites in non infected and SFV-h.P_{2X} infected CHO-K1 cells compared with those in rat vas deferens

Compound	*Non infected CHO-K1 cells*		*SFV-P_{2X} infected CHO-K1 cells*		*Vas deferens*	
	pIC_{50}	*Hill slope*	*pIC_{50}*	*Hill slope*	*pIC_{50}*	*Hill slope*
ATP	6.82 ± 0.13	0.77 ± 0.08	8.93 ± 0.05	1.03 ± 0.04	9.01 ± 0.08	0.85 ± 0.10
ATPγS	7.06 ± 0.10	0.75 ± 0.02	8.08 ± 0.03	0.87 ± 0.07	8.73 ± 0.10	0.89 ± 0.04
2-m2-S-ATP	5.43 ± 0.14	0.64 ± 0.07	8.23 ± 0.14	0.80 ± 0.06	8.79 ± 0.12	0.68 ± 0.03
$\alpha\beta$meATP	4.84 ± 0.34	0.54 ± 0.09	7.17 ± 0.09	0.91 ± 0.06	7.57 ± 0.14	0.92 ± 0.06
ADP	6.10 ± 0.09	0.91 ± 0.06	7.27 ± 0.04	0.92 ± 0.14	7.18 ± 0.13	0.96 ± 0.13
$\beta\gamma$meATP	5.01 ± 0.31	0.64 ± 0.07	6.25 ± 0.08	0.84 ± 0.09	7.24 ± 0.11	0.84 ± 0.04
L-$\beta\gamma$meATP	3.44 ± 0.20	0.77 ± 0.15	5.39 ± 0.04	0.65 ± 0.07	5.83 ± 0.09	0.88 ± 0.06
$\alpha\beta$meADP	3.99 ± 0.29	0.55 ± 0.16	4.22 ± 0.04	0.75 ± 0.13	4.36 ± 0.11	0.83 ± 0.07

Competition binding studies on the sites labelled by 0.15 nM [^{35}S]-ATPγS in membranes prepared from non infected CHO-K1 cells, CHO-K1 cells infected with the human bladder P_{2x} purinoceptor using Semliki Forest virus (SFV-h.P_{2x}) and rat vas deferens. The data are the mean ± s.e.mean of 3–5 determinations.

Table 2 Competition studies at the [^{33}P]-ATP binding sites in non infected and SFV-h.P_{2x} infected CHO-K1 cells compared with those in rat vas deferens

Compound	*Non infected CHO-K1 cells*		*SFV-h.P_{2x} infected CHO-K1 cells*		*Vas deferens*	
	pIC_{50}	*Hill slope*	*pIC_{50}*	*Hill slope*	*pIC_{50}*	*Hill slope*
ATP	7.15±0.29	0.72±0.06	9.16±0.05	1.02±0.02	9.19±0.04	0.91±0.10
ATPγS	6.89±0.16	0.78±0.04	8.09±0.05	0.96±0.03	8.60±0.13	0.99±0.11
2-me-S-ATP	4.39±0.29	0.34±0.02	8.37±0.08	1.01±0.01	8.78±0.18	0.81±0.02
αβmeATP	5.73±0.05	0.76±0.06	7.55±0.08	0.96±0.01	7.64±0.13	0.90±0.04
ADP	5.94±0.18	0.77±0.08	7.37±0.06	0.96±0.05	7.26±0.18	0.89±0.05
βγmeATP	5.66±0.11	0.47±0.09	6.29±0.09	0.97±0.01	7.32±0.09	0.92±0.07
L-βγmeATP	3.47±0.23	0.66±0.19	5.58±0.06	0.71±0.05	5.80±0.10	0.81±0.02
αβmeADP	3.90±0.25	0.87±0.21	4.21±0.15	0.71±0.11	4.30±0.16	0.89±0.02

Competition binding studies on the sites labelled by 0.1 nM [^{33}P]-ATP in membranes prepared from non infected CHO-K1 cells, CHO-K1 cells infected with the human bladder P_{2x} purinoceptor using Semliki Forest virus (SFV-h.P_{2x}) and rat vas deferens. The data are the mean±s.e.mean of 3–5 determinations.

In the SFV-h.P_{2x} infected CHO-K1 cells, but not in SFV-lacZ infected cells, there was a marked increase in high affinity specific binding of [^{3}H]-αβmeATP and [^{35}S]-ATPγS when compared to non infected CHO-K1 cells. This would suggest that infection by SFV *per se* was not responsible for the increase in high affinity radioligand binding. Furthermore, the infection of CHO-K1 cells with SFV-h.P_{2x} not only increased specific binding of [^{3}H]-αβmeATP and [^{35}S]-ATPγS but also changed the binding characteristics of the sites labelled, again indicating that infection had not simply increased expression of the endogenous binding sites. Since electrophysiological studies on these cells have demonstrated that non infected and SFV-lacZ infected CHO-K1 cells do not possess an electrophysiologically defined P_{2x} purinoceptor, but that following the SFV-h.P_{2x} infection ATP-evoked currents can be measured, the simplest interpretation of these results is that [^{3}H]-αβmeATP and [^{35}S]-ATPγS do label the recombinant P_{2x} purinoceptor in the SFV-h.P_{2x} infected cells.

The demonstration that [^{3}H]-αβmeATP can label the human bladder recombinant P_{2x} purinoceptor with high affinity was of interest in view of previous studies using this radioligand (Bo *et al.*, 1992; Michel & Humphrey, 1993). It has been suggested that [^{3}H]-αβmeATP binding sites in rat vas deferens are P_{2x} purinoceptors although proving this has been complex given the lack of suitable antagonists and the stability problems associated with the use of agonists (see Introduction). While the present findings do not suggest that all high affinity [^{3}H]-αβmeATP binding sites are P_{2x} purinoceptors they do provide direct evidence that [^{3}H]-αβmeATP can label the P_{2x} purinoceptor with high affinity. Furthermore, since affinity estimates for αβmeATP (8.2), L-βγmeATP (7.2) and αβmeADP (4.3) at the human bladder recombinant P_{2x} purinoceptor labelled with [^{3}H]-αβmeATP were very similar to the respective values of 8.4 (Michel & Humphrey, 1993), 7.7 (Michel *et al.*, 1994) and 3.2 (Michel *et al.*, 1995) obtained at the high affinity binding sites labelled by [^{3}H]-αβmeATP in rat vas deferens, it is likely that the high affinity [^{3}H]-αβmeATP binding sites in vas deferens are P_{2x} purinoceptors.

The additional demonstration that [^{35}S]-ATPγS can be used to label the human bladder recombinant P_{2x} purinoceptor enables a more definitive interpretation of our previous studies on the binding of this radioligand to rat vas deferens membranes. We have shown that, in the absence of divalent cations, [^{35}S]-ATPγS labels high affinity binding sites in rat vas deferens membranes (Michel & Humphrey, 1996). However, the rank order of agonist affinity in those binding studies did not agree with that obtained at the [^{3}H]-αβmeATP binding sites, labelled in the presence of calcium ions, or with the currently accepted profile of the P_{2x} purinoceptor obtained in functional studies on the rat vas deferens, since 2-meS-ATP, ATPγS and ATP were more potent competing ligands than αβmeATP (Michel & Humphrey, 1996). Given the present demonstration that [^{35}S]-ATPγS can label the human bladder recombinant P_{2x} purinoceptor with high affinity, and the finding that affinity estimates for a number of compounds in competition studies on the [^{35}S]-ATPγS binding sites in the rat vas deferens and the human bladder recombinant P_{2x} purinoceptor are similar, it seems likely that the [^{35}S]-ATPγS binding sites in rat vas deferens are indeed P_{2x} purinoceptors. This would also suggest that the unusual rank order of agonist potencies obtained in binding studies with [^{35}S]-ATPγS is a consequence of the assay being performed in the absence of divalent cations as suggested previously (Michel & Humphrey, 1996).

It should be noted that there were some differences in affinity estimates obtained at the [^{35}S]-ATPγS binding sites in rat vas deferens and at the human bladder recombinant P_{2x} purinoceptor, notably approximately ten fold for βγmeATP. This could indicate either a difference between bladder and vas deferens P_{2x} purinoceptors or a species difference in the binding characteristics of the P_{2x} purinoceptor. The former possibility seems unlikely since binding studies have failed to reveal any significant difference between the rat bladder and rat vas deferens [^{35}S]-ATPγS binding sites (unpublished observations). Furthermore, there are high levels of the mRNA for the P_{2x} purinoceptor subtype cloned from rat vas deferens in rat bladder (Valera *et al.*, 1994) suggesting that the two tissues express the same P_{2x} purinoceptor. Thus, it is more likely that there is a species difference between the rat and human P_{2x} purinoceptors despite their high homology (89% identity; 94% similarity). This issue could be resolved by performing binding studies on the rat vas deferens P_{2x} purinoceptor expressed in CHO-K1 cells, which may also help to determine if the cell expression system is an important factor, or by performing binding studies in human bladder membranes.

While the main finding of this paper is that [^{3}H]-αβmeATP and [^{35}S]-ATPγS can be used to label the human bladder P_{2x} purinoceptor, these results confirm previous observations that in binding studies, P_{2x} purinoceptor agonists possess nanomolar affinity for the P_{2x} purinoceptor (Bo & Burnstock, 1990; Bo *et al.*, 1992; Michel & Humphrey, 1996). This contrasts with their low potency in functional studies. Thus, even in electrophysiological studies, where agonists are applied by a concentration-clamp technique to circumvent any potential problems of nucleotide metabolism, the EC_{50} values for αβ meATP and ATP are micromolar rather than nanomolar at both the endogenous vas deferens P_{2x} purinoceptor and the vas deferens recombinant P_{2x} purinoceptor expressed in oocytes (Valera *et al.*, 1994; Khakh *et al.*, 1995b). Perhaps, the discrepancy between binding affinity and functional potency reflects binding of the radioligands to a desensitized state of the P_{2x} purinoceptor as previously suggested (Michel & Humphrey, 1993).

The results of this study are also consistent with our pre-

vious observation that affinity estimates determined in binding studies on the P_{2X} purinoceptor are markedly affected by divalent cations. In the present study, $\alpha\beta$meATP and L-$\beta\gamma$meATP possessed high affinity for the recombinant human bladder P_{2X} purinoceptor when labelled with [^{3}H]-$\alpha\beta$meATP in the presence of calcium ions but possessed lower affinity when the recombinant receptor was labelled by [^{35}S]-ATPγS in the absence of divalent cations. This effect of divalent cations on affinity estimates for $\alpha\beta$meATP and L-$\beta\gamma$meATP was similar to that observed for rat vas deferens membranes (Michel & Humphrey, 1995). Our previous studies in rat vas deferens membranes also indicated that ATP, ATPγS and 2-meS-ATP possess much higher affinity for the [^{35}S]-ATPγS and [^{3}H]-$\alpha\beta$ meATP binding sites labelled in the absence of divalent cations than for the [^{3}H]-$\alpha\beta$meATP binding sites labelled in the presence of calcium ions (Michel & Humphrey, 1993; 1996).

A number of possible explanations has been suggested for the effect of calcium on nucleotide affinity estimates, including the possibilities that metabolism of nucleotides in the presence of calcium leads to an underestimation of their true affinity, that calcium ions are required for high affinity binding of some nucleotide analogues to a desensitized state of the P_{2X} purinoceptor or that the tetrabasic form of those ionisable analogues of ATP is the preferred agonist state (Michel & Humphrey, 1996). The availability of recombinant receptor should now enable these various possibilities to be examined further.

Finally, in the absence of divalent cations, ATP possesses the highest affinity, of any of the nucleotides examined, for the human bladder recombinant P_{2X} purinoceptor. Consistent with its high affinity, [α^{33}P]-ATP, in the absence of divalent cations, could also be used to label the human bladder recombinant P_{2X} purinoceptor and affinity estimates obtained with this radioligand were almost identical to those obtained with [^{35}S]-ATPγS in both the SFV-h.P_{2X} infected CHO-K1 cells and in the rat vas deferens membranes.

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

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(Received July 20, 1995
Revised November 6, 1995
Accepted November 13, 1995)