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High affinity P_{2X}-purinoceptor binding sites for [^{35}S]-adenosine 5'-O-[3-thiotriphosphate] in rat vas deferens membranes

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1 The binding sites labelled by [^{35}S]-adenosine 5'-O-[3-thiotriphosphate]([^{35}S]-ATPγS) at 4°C in rat vas deferens membranes were studied and compared to the sites labelled by [^{3}H]-α,β-methylene ATP ([^{3}H]-αβmeATP) to ascertain whether [^{35}S]-ATPγS can be used to label the P_{2X} purinoceptor.

2 In the presence of 4 mM $CaCl_2$, the binding of 0.2 nM [^{35}S]-ATPγS to vas deferens membranes was increased 3.4 fold, when compared to studies performed in the absence of calcium. However, binding did not appear to be solely to P_{2X} purinoceptors since [^{35}S]-ATPγS labelled a heterogeneous population of sites and about 72% of the sites possessed high affinity ($pIC_{50}=7.5$) for guanosine 5'-O-[3-thiotriphosphate] (GTPγS). Even in the presence of 1 μM GTPγS, to occlude the sites with high affinity for GTPγS, the binding of [^{35}S]-ATPγS was heterogeneous and since there was also evidence of extensive metabolism of ATP in the presence of calcium, the binding of [^{35}S]-ATPγS under these conditions was not studied further.

3 In the absence of calcium ions, [^{35}S]-ATPγS bound to a single population of sites ($pK_D=9.23$; $B_{max}=4270$ fmol mg^{-1} protein). Binding reached steady state within 3 h ($t_{\frac{1}{2}}=38$ min), was stable for a further 4 h and was readily reversible upon addition of 10 μM unlabelled ATPγS ($t_{\frac{1}{2}}=45$ min). In competition studies the binding of 0.2 nM [^{35}S]-ATPγS was inhibited by a number of P_{2X} purinoceptor agonists and antagonists, but not by adenosine receptor agonists, staurosporine (1 μM) or several ATPase inhibitors. The rank order of agonist affinity estimates (pIC_{50} values) in competing for the [^{35}S]-ATPγS binding sites was: ATP (9.01), 2-methylthio- ATP (8.79), ATPγS (8.73), αβmeATP (7.57), ADP (7.24), β,γ-methylene ATP (7.18), L-β,γ-methylene ATP (5.83), α,β-methylene ADP (4.36).

4 Affinity estimates (pIC_{50} values) for the P_{2X} purinoceptor antagonists, suramin (5.20), pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (4.23), pyridoxal 5-phosphate (3.42), cibacron blue (5.70) and Evan's blue (5.79) were broadly similar to those obtained at the [^{3}H]-αβmeATP binding sites in vas deferens. However, ATP, 2-methylthio-ATP, ATPγS and ADP displayed 17-512 fold higher affinity for the [^{35}S]-ATPγS, than for the [^{3}H]-αβmeATP binding sites, whereas αβmeATP and L-β,γ-methylene ATP displayed 5 and 28 fold, respectively, higher affinity for the [^{3}H]-αβmeATP than for the [^{35}S]-ATPγS binding sites.

5 The differences in agonist affinity for the [^{35}S]-ATPγS and [^{3}H]-αβmeATP binding sites probably reflect the fact that the former sites were labelled in the absence of calcium, while the latter sites were labelled in its presence. This could differentially affect ionisation state and/or metabolism of the nucleotides when using the two radioligands. Since affinity estimates for ATP, 2-methylthio-ATP, ATPγS, αβmeATP and L-β,γ-methylene ATP were different when calcium ions were omitted in studies using [^{3}H]-αβmeATP but similar to the affinity estimates obtained at the [^{35}S]-ATPγS binding sites labelled in the absence of calcium, it is likely that [^{35}S]-ATPγS and [^{3}H]-αβmeATP label the same sites in rat vas deferens.

6 We conclude that, in the absence of divalent cations, [^{35}S]-ATPγS labels P_{2X} purinoceptors in rat vas deferens and as such may represent a new, high specific activity, radioligand for the study of such receptors.

Keywords: [^{35}S]-ATPγS; rat vas deferens; P_{2X} purinoceptor

Introduction

It is now well established from extensive functional studies (see Abbrachio *et al.*, 1993; Fredholm *et al.*, 1994) and more recently molecular biological studies (Webb *et al.*, 1993; Lustig *et al.*, 1993; Valera *et al.*, 1994; Brake *et al.*, 1994) that specific membrane receptors exist for extracellular ATP. At present at least five distinct subtypes have been identified, namely the P_{2X} P_{2Y} P_{2Z} P_{2T} and P_{2U} purinoceptors (Abbrachio *et al.*, 1993; Fredholm *et al.*, 1994). Furthermore, there is heterogeneity of both the P_{2X} and P_{2Y} purinoceptors (Barnard *et al.*, 1994; Valera *et al.*, 1994; Brake *et al.*, 1994; Burnstock *et al.*, 1994; Abbrachio & Burnstock, 1994). While the P_{2X} purinoceptors have been extensively characterized in functional studies, their ligand binding properties have been poorly characterized. This has been due both to the lack of specific, high affinity P_{2X} purinoceptor antagonists and also the potential difficulties of using agonist radioligands which, in many cases, are metabolically unstable.

An important advance in the study of the ligand binding properties of the P_{2X} purinoceptor was the introduction of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,βmeATP) as a radioligand (Bo & Burnstock, 1989; 1990). The binding sites for this radioligand have now been studied in detail in a number of tissues and the available evidence suggests that the high affinity binding sites for [^{3}H]-αβmeATP represent P_{2X} purinoceptors. Thus, the tissue distribution of the high affinity [^{3}H]-αβmeATP binding sites is consistent with that expected of the P_{2X} pur-

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inoceptor since there is a high density of the sites in rat bladder (Bo & Burnstock, 1990) and vas deferens (Bo *et al.*, 1992), two tissues used extensively for functional studies on the P_{2X} purinoceptor (Burnstock & Kennedy, 1985). Furthermore, studies with agonists (Bo *et al.*, 1992) and antagonists (Khakh *et al.*, 1994), have provided evidence that high affinity [^{3}H]-αβmeATP binding sites do represent P_{2X} purinoceptors. The binding characteristics of [^{3}H]-αβmeATP are complex, with more recent evidence suggesting that there may be heterogeneity of the high affinity [^{3}H]-αβmeATP binding sites (Michel *et al.*, 1994). Furthermore, the presence of low affinity [^{3}H]-α,βmeATP binding sites in many tissues can complicate studies with this radioligand (Michel & Humphrey, 1993). In addition it has been observed that while most P_{2X} purinoceptor agonists possess nanomolar affinity for the [^{3}H]-αβmeATP binding sites, these agonists activate the P_{2X} purinoceptor at micromolar concentrations in functional studies (Michel & Humphrey, 1993).

In preliminary studies we found that, in the absence of divalent cations, the affinity of adenosine 5'-O-[3-thiotriphosphate] (ATPγS) increased (unpublished observation) while that of αβmeATP decreased (Michel & Humphrey, 1994). Furthermore, ATPγS has been shown to possess very high affinity for the [^{3}H]-αβmeATP binding sites in rat bladder when studied in the absence of divalent cations (Bo *et al.*, 1994). These observations suggest that, in the absence of divalent cations, ATPγS may be suitable for use as a radioligand in studies on the P_{2X} purinoceptor. Since [^{35}S]-ATPγS is commercially available, we have studied the binding of this radioligand to rat vas deferens membranes to determine whether [^{35}S]-ATPγS can be used as a high specific activity radioligand for the study of the P_{2X} purinoceptor. Rat vas deferens membranes were chosen because previous studies have demonstrated that this tissue possesses functional P_{2X} purinoceptors which [^{3}H]-αβmeATP labels (see above). A preliminary account of these studies has been presented to the British Pharmacological Society (Michel & Humphrey, 1995).

Methods

Receptor binding studies

Membrane preparation procedures and binding assay conditions were essentially identical to those employed for the study of [^{3}H]-αβmeATP binding to rat vas deferens (Michel & Humphrey, 1994) with a few minor modifications. In the majority of studies a divalent cation free 50 mM Tris, 1 mM EDTA assay buffer, pH 7.4 at 4°C, was used although in some studies 4 mM $CaCl_2$ was also included since previous studies on the P_{2X} purinoceptor using [^{3}H]-αβmeATP have been performed under such conditions (Michel & Humphrey, 1994). Non-specific binding (NSB) of the radioligand was defined by use of 10 μM ATPγS. Incubations were conducted (usually for 3 h) in a final assay volume of 250 μl at 4°C and were terminated by vacuum filtration over wet, 4°C 20 mM $Na_4P_2O_7$ pretreated GF/B glass fibre filters using a Brandel 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.

The binding of [^{3}H]-αβmeATP to rat vas deferens in 50 mM Tris 1 mM EDTA buffer was performed as described above with the exceptions that a 40 min incubation period was employed, the radioligand concentration was 10 nM and NSB was defined by use of 30 μM αβmeATP.

In competition studies, the ability of a series of nucleotide analogues to compete for the binding sites, labelled by either 0.2 nM [^{35}S]-ATPγS or 10 nM [^{3}H]-αβmeATP, were determined over a range of concentrations spanning at least five log units as described previously (Michel & Humphrey, 1994). In saturation studies, total binding and NSB of [^{35}S]-ATPγS were determined in triplicate and duplicate, respectively, over a radioligand concentration range of 0.05–10 nM. In kinetic association studies, total binding and NSB of 0.2 nM [^{35}S]-ATPγS were measured in triplicate over a 8 h period. In dissociation studies, 10 μM was added to the assay tubes 40 min after initiating association and both total and NSB were measured for a further 6 h. Protein was measured by the dye binding method (Biorad).

Determination of ATPase activity

ATPase activity was determined using a modified version of the procedure described by Beukers *et al.* (1993), in which the production of the radiolabelled γ phosphate from [γ^{33}P]-ATP was measured. The vas deferens membranes, competing compounds and 0.2 nM [γ^{33}P]-ATP were incubated in 250 μl of a 50 mM Tris, 1 mM EDTA assay buffer (pH 7.4) for 10 min at 4°C either in the presence or absence of 4 mM $CaCl_2$. The reaction was terminated by addition of 500 μl of charcoal suspension (2 g charcoal to 50 ml of 0.1 N HCl) and the assay tubes were centrifuged at 1500 *g* for 45 min. A 200 μl aliquot of the supernatant was removed and the radioactivity present in this aliquot, and which is assumed to represent the ^{33}P-labelled inorganic phosphate liberated from ATP, was determined by liquid scintillation spectrophotometry.

Data analysis

Saturation binding data were analysed by LIGAND (Munson & Rodbard, 1980) as described previously (Michel & Humphrey, 1994). Competition data were analysed by iterative curve-fitting techniques (Michel & Whiting, 1984) to determine the IC_{50} and Hill slope (n_H) for the competition curve. When the Hill slope of the competition curve was less than unity the data were further analysed to determine if they could be better described by assuming the compounds to be competing with two populations of specific binding sites. For this analysis the IC_{50} for each site and the proportion of high and low affinity sites was determined (Michel & Whiting, 1984).

In kinetic studies the observed rate constant for association (K_{obs}) and the rate constant for dissociation (K_{-1}) were determined by iterative curve fitting procedures (SIGMAPLOT, Jandel Scientific). The rate constant for association K_{+1} was calculated from the expression $K_{+1}=(K_{obs}-K_{-1})/[L]$, where [L] represents the radioligand concentration.

The IC_{50} values were not adjusted to take account of the presence of radioligand since, in the presence of calcium ions, binding of the radioligand was heterogeneous (see Results). However, in the absence of divalent cations, with a radioligand K_D of 0.59 nM (see Results) and a radioligand concentration of 0.2 nM then it is likely that IC_{50} values will be approximately 1.3 fold greater than the K_i (Cheng & Prusoff, 1973). All IC_{50} values are presented as the negative logarithm of the IC_{50} (pIC_{50}). Unless otherwise stated the data presented are the mean ± s.e.mean of between 3 and 5 separate experiments.

Materials

Staurosporine, Evan's blue, pyridoxal 5'-phosphate, sodium azide (NaN_3), guanosine 5'-O-[3-thiotriphosphate] (GTPγS) and S-(*p*-nitrobenzyl)-6-thio-inosine (NBTI) were obtained from Sigma. L-β,γ-methylene ATP sodium salt (L-βγmeATP) was obtained from Research Biochemicals while pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) was from Tocris Cookson. Charcoal was obtained from Norit. All other drugs were obtained as described previously (Michel & Humphrey, 1994).

With the exception of ouabain, cyclopropyladenosine, adenosine and NBTI, the compounds were dissolved in assay buffer. Ouabain, thapsigargin, staurosporine and NBTI were dissolved in dimethylsulphoxide (DMSO), cyclopropyladenosine and adenosine in 1 M HCl while cyclopiazonic acid was dissolved in 50 mM Tris base. In separate studies it was shown

that these vehicles neither affected specific binding of the radioligands nor ATPase activity. [^{35}S]-ATPγS (specific activity 1500 Ci mmol^{-1}), [^{3}H]-αβ-meATP (specific activity 28 Ci mmol^{-1}), and [γ^{33}P]-ATP (specific activity 3000 Ci mmol^{-1}) were obtained from Amersham, UK.

Results

Kinetics of [^{35}S]-ATPγS binding in the absence of divalent cations

Binding of 0.2 nM [^{35}S]-ATPγS to vas deferens membranes achieved a steady state within 120–180 min ($t_{½}$ = 37.8 ± 6.1 min; n = 3) and was stable for at least a further 4–5 h. The binding of [^{35}S]-ATPγS was reversible following addition of 10 μM ATPγS ($t_{½}$ = 45.3 ± 8.1 min) with >90% of the specifically bound [^{35}S]-ATPγS dissociating within 7 h (Figure 1). These kinetic data could be best described by assuming that the radioligand was associating with and dissociating from a single population of non-interacting binding sites. The values for K_{+1} and K_{-1} calculated from this analysis were 7.196 ± 1.009 × 10^{7} M min^{-1} and 0.016 ± 0.003 min^{-1}, respectively. The kinetically derived K_D was 0.24 nM, equivalent to a pK_D of 9.62.

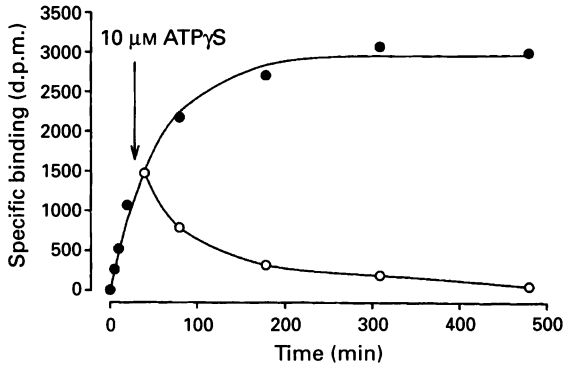

Figure 1 Kinetics of [^{35}H]-ATPγS binding in rat vas deferens membranes. Specific binding of 0.2 nM [^{35}S]-ATPγS was measured for up to 480 min after initiation of binding (●). Rat vas deferens membranes and 0.2 nM [^{35}S]-ATPγS were incubated for 40 min before addition of 10 μM ATPγS and specific binding was measured for up to 440 min thereafter (○). The data are from a single representative experiment. For each data point non specific binding, which represented between 170 and 230 d.p.m., was subtracted.

Saturation binding of [^{35}S]-ATPγS in the absence of divalent cations

In saturation studies (Figure 2a), [^{35}S]-ATPγS bound to a single population of non-interacting binding sites with a pK_D of 9.23 ± 0.08 and B_{max} of 4270 ± 460 fmol mg^{-1} protein. Although there does appear to be slight curvature in the Scatchard plot of the specific binding data (Figure 2b), and the data could be analysed assuming the presence of a second non-saturable component of binding, this more complex model did not represent a statistically significant better fit than the single site model.

Characterization of the [^{35}S]-ATPγS binding sites labelled in the absence of divalent cations

The binding of 0.2 nM [^{35}S]-ATPγS was inhibited by a number of nucleotide analogues. With the exception of the low affinity compounds, αβmeADP and L-βγmeATP, binding was inhibited to the same extent by all compounds (Table 1 and Figure 3a). For most compounds, the Hill slope was close to unity and in all cases the data could be best described by assuming competition with a single population of specific [^{35}S]-ATPγS binding sites. The Hill slope for 2-meS-ATP was less than unity but the data could only be fitted to a model assuming the presence of a single population of sites. ATP, 2-meS-ATP and ATPγS were the most potent competing com-

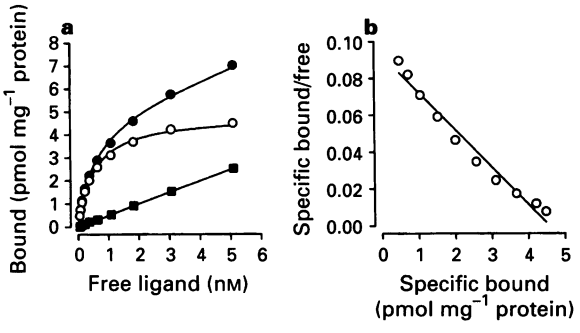

Figure 2 Saturation binding of [^{35}S]-ATPγS to rat vas deferens membranes. (a) Total binding (●), specific binding (○) or non specific binding (■) were measured at radioligand concentrations between 0.05 and 5.2 nM. (b) The specific binding data from (a) are presented as a Scatchard plot. The data are from a single representative experiment.

Table 1 Competition for [^{35}S]-ATPγS binding sites in rat vas deferens membranes determined under different experimental conditions

Compound	*Ca-free*		*4mM $CaCl_2$*		*4 mM $CaCl_2$ and 1 μM GTPγS*	
	pIC_{50}	*n_H*	*pIC_{50}*	*n_H*	*pIC_{50}*	*n_H*
ATP	9.01 ± 0.08	0.85 ± 0.10	6.84 ± 0.04	0.50 ± 0.01	6.84 ± 0.07	0.54 ± 0.01
ATPγS	8.73 ± 0.10	0.89 ± 0.04	8.16 ± 0.01	0.69 ± 0.11	7.82 ± 0.30	0.64 ± 0.04
2-meS-ATP	8.79 ± 0.12	0.68 ± 0.03	6.69 ± 0.15	0.45 ± 0.01	6.06 ± 0.27	0.42 ± 0.10
αβmeATP	7.57 ± 0.14	0.92 ± 0.06	6.04 ± 0.36	0.54 ± 0.04	6.50 ± 0.16	0.38 ± 0.06
βγmeATP	7.18 ± 0.13	0.96 ± 0.13	4.85 ± 0.44	0.38 ± 0.02	5.83 ± 0.21	0.36 ± 0.07
ADP	7.24 ± 0.11	0.84 ± 0.04	6.10 ± 0.06	0.60 ± 0.05	6.07 ± 0.18	0.79 ± 0.20
L-βγmeATP	5.83 ± 0.09	0.88 ± 0.06	4.72 ± 0.18	0.42 ± 0.12	4.96 ± 0.09	0.35 ± 0.09
αβmeADP	4.36 ± 0.11	0.83 ± 0.07	4.48 ± 0.18	0.54 ± 0.05	4.36 ± 0.13	1.02 ± 0.05
Suramin	5.20 ± 0.10	0.69 ± 0.06	ND	ND	ND	ND
Cibacron Blue	5.70 ± 0.04	1.21 ± 0.09	ND	ND	ND	ND
PPADS	4.23 ± 0.05	0.74 ± 0.02	ND	ND	ND	ND
Pyridoxal 5-phosphate	3.42 ± 0.02	0.87 ± 0.01	ND	ND	ND	ND
Evan's blue	5.79 ± 0.05	0.91 ± 0.04	ND	ND	ND	ND
DIDS	4.75 ± 0.04	0.86 ± 0.05	ND	ND	ND	ND

The data are the mean ± s.e.mean of 3–5 determinations for the pIC_{50} and the Hill slope (n_H). Competition studies were carried out for 3 h at 4°C using a radioligand concentration of 0.2 nM in a 50 mM Tris, 1 mM EDTA assay buffer either in the absence or presence of 4 mM $CaCl_2$. In some studies performed in the presence of 4 mM $CaCl_2$, 1 μM GTPγS was also included. ND = not determined.

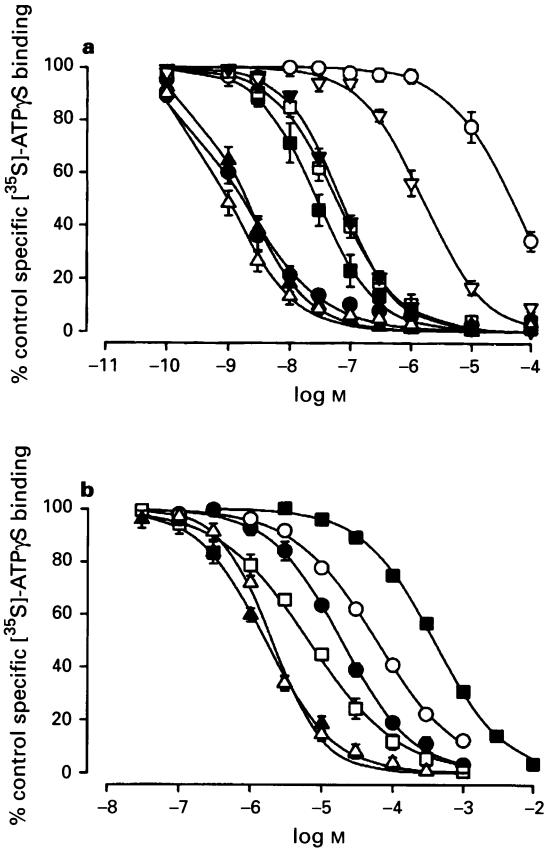

Figure 3 Competition for [^{35}S]-ATPγS binding sites in rat vas deferens membranes by nucleotides and P_{2X} purinoceptor antagonists. (a) Competition by ATP (△), 2-meS-ATP (●), ATPγS (▲), αβmeATP (■), ADP (□), βγmeATP, (▼), L-βγmeATP (▽) and αβmeADP (○). (b) Competition by cibacron blue (△), Evan's blue (▲), suramin (□), DIDS (●), PPADS (○) and pyridoxal 5'phosphate (■). The data are the mean±s.e.mean of 3–5 determinations.

pounds with pIC_{50} values of 9.01, 8.79 and 8.73, respectively, while αβmeATP, ADP and βγmeATP were about 12–33 fold less potent than ATP. [^{35}S]-ATPγS binding was inhibited only with low affinity by GTPγS ($pIC_{50}=5.4\pm0.21$; $n_H=0.89\pm0.12$), while the following compounds did not affect binding at the concentrations indicated in parentheses: adenosine (100 μM), AMP (100 μM), sodium thiosulphite (1 mM), sodium azide (1 mM), staurosporine (1 μM), NECA (10 μM), cyclopropyladenosine (10 μM), cyclopiazonic acid (10 μM).

A number of P_{2X} purinoceptor antagonists, including cibacron blue, suramin, Evan's blue, PPADS, DIDS and pyridoxal 5'phosphate inhibited binding (Table 1, Figure 3b). For these compounds the Hill slopes were close to unity (0.69–1.21) and the data for all compounds could only be described by assuming competition with a single population of [^{35}S]-ATPγS binding sites.

Effect of calcium on [^{35}S]-ATPγS binding sites in rat vas deferens

Previous studies demonstrated a marked effect of calcium ions on [^{3}H]-αβmeATP binding to rat vas deferens (Michel & Humphrey, 1994). In studies using [^{35}S]-ATPγS, 4 mM $CaCl_2$ increased binding by 3.4±0.4 fold ($n=3$) when compared to binding measured in the absence of calcium. Under these conditions the Hill slopes of the competition curves to all P_{2X} purinoceptor agonists were less than unity and the potency of most nucleotides, apart from ATPγS and αβmeADP, decreased at least 10 fold when compared to affinity estimates obtained in the absence of calcium ions (Table 1). In the presence of 4 mM $CaCl_2$, GTPγS was a very potent inhibitor of binding ($pIC_{50}=7.2\pm0.2$, $n_H=0.43\pm0.05$, $n=3$) competing for 72.4±3.7% of the sites with high affinity (pIC_{50} of 7.5±0.1) and the remaining sites with a pIC_{50} of 5.4±0.2 (Figure 4a). A limited series of experiments was performed in the presence of 1 μM GTPγS in order to occlude those [^{35}S]-ATPγS binding sites with high affinity for GTPγS. Under these conditions, however, the Hill slopes of the competition curves for most agonists were still less than unity and the affinity estimates lower than obtained in the absence of divalent cations (Table 1).

Metabolism of ATP in the presence of calcium

To investigate the possibility that ATP may be differentially metabolised in the presence and absence of calcium ions, the breakdown of [γ^{33}P]-ATP was measured. At 4°C there was a pronounced metabolism of ATP in the presence of 4 mM $CaCl_2$ and this breakdown of ATP was eliminated when $CaCl_2$ was omitted and 1 mM EDTA included in the assay (Figure 4b).

Comparison of the sites labelled by [^{3}H]-αβmeATP and [^{35}S]-ATPγS in rat vas deferens

In our previous studies we have examined [^{3}H]-αβmeATP binding in the presence of calcium ions. To compare the sites labelled by [^{3}H]-αβmeATP and [^{35}S]-ATPγS under similar conditions, the [^{3}H]-αβmeATP binding sites were characterized in the absence of divalent cations. The affinity estimates of a number of compounds under these conditions were very different from those determined in the presence of calcium ions (see Figure 5). For most compounds, apart from L-βγmeATP and αβmeADP, the Hill slopes obtained in the absence of divalent cations were less than unity and the data were best described by assuming that the compounds could identify two

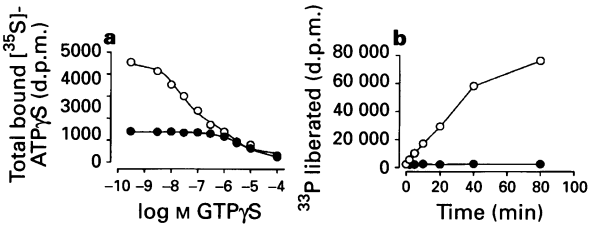

Figure 4 The effect of calcium on [^{35}S]-ATPγS binding and ATP metabolism in rat vas deferens at 4°C. The data are from single representative experiments performed using a 50 mM Tris, 1 mM EDTA assay buffer (●) or a 50 mM Tris 1 mM EDTA, 4 mM $CaCl_2$ assay buffer (○). (a) Competition by GTPγS for [^{35}S]-ATPγS binding. (b) Production of ^{33}P inorganic phosphate from 0.1 nM [γ^{33}P]-ATP.

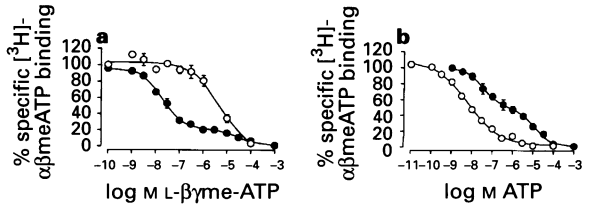

Figure 5 The effect of calcium on the competition by L-βγmeATP and ATP for the [^{3}H]-αβmeATP binding sites in rat vas deferens. The binding of 1 nM [^{3}H]-αβmeATP was measured in a 50 mM Tris 1 mM EDTA, 4 mM $CaCl_2$ assay buffer (●) while the binding of 10 nM [^{3}H]-αβmeATP was determined in a 50 mM Tris, 1 mM EDTA assay buffer (○). (a) Competition by L-βγmeATP. (b) Competition by ATP. The data are the mean±s.e.mean of 3–5 determinations. The competition studies performed in the presence of 4 mM $CaCl_2$ have been published previously (Michel & Humphrey, 1993; Trezise *et al.*, 1995).

Table 2 Competition for [^{3}H]-αβmeATP binding sites of rat vas deferens membranes studied in the absence of divalent cations

Compound	Hill model		Two site model			
			Site 1		Site 2	
	pIC_{50}	n_H	pIC_{50}	% of sites	pIC_{50}	% of sites
ATP	8.05±0.11	0.60±0.03	8.45±0.13	74.5±1.6	6.31±0.30	25.5±1.6
ATPγS	8.02±0.18	0.60±0.07	8.40±0.10	71.2±2.9	6.54±0.16	28.8±2.9
2-meS-ATP	7.93±0.12	0.65±0.04	8.36±0.09	72.8±3.6	6.28±0.31	27.2±3.6
αβmeATP	7.53±0.08	0.68±0.03	7.81±0.12	73.6±7.3	6.02±0.39	26.4±7.3
βγmeATP	6.49±0.06	0.71±0.01	6.95±0.12	77.7±1.4	5.40±0.08	22.3±1.4
ADP	6.48±0.15	0.78±0.15	6.92±0.13	67.9±5.5	5.58±0.12	32.1±5.5
L-βγmeATP	5.33±0.10	0.93±0.13	5.36±0.13	100	ND	ND
αβmeADP	4.16±0.20	1.11±0.13	4.20±0.21	100	ND	ND

Incubations were carried out over a 40 min period at 4°C using a radioligand concentration of 10 nM. The data are the mean ± s.e.mean of 3–5 determinations. Competition curves were first analysed according to a Hill Model in which the pIC_{50} and Hill slope (n_H) were determined. In addition the data were also analysed assuming competition with either one or two populations of specific binding sites. When the two site model represented a significantly better fit than the one site model the data have been presented as the pIC_{50} value for each site and the % of specific binding sites that each site comprised. The site for which the compounds possessed highest affinity was designated as site 1. ND indicates that data could not be fitted to a two site model.

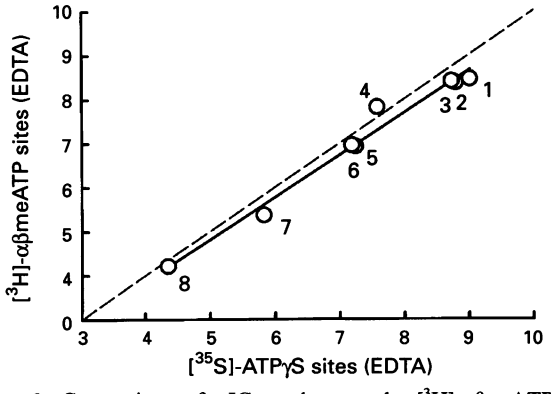

Figure 6 Comparison of pIC_{50} values at the [^{3}H]-αβmeATP and [^{35}S]-ATPγS binding sites in rat vas deferens. pIC_{50} values for [^{35}S]-ATPγS are from Table 1 while pIC_{50} values for [^{3}H]-αβmeATP are for site 1 from Table 2. The nucleotides were: (1) ATP, (2) 2-meSATP, (3) ATPγS, (4) αβmeATP, (5) ADP, (6) βγmeATP, (7) L-βγmeATP and (8) αβmeADP.

populations of specific [^{3}H]-αβmeATP binding site (Table 2). The affinity estimates determined at the [^{3}H]-αβmeATP binding sites in the absence of divalent cations (i.e. pIC_{50} for site 1 in Table 2) were similar to the respective affinity estimates for the [^{35}S]-ATPγS binding sites studied in the absence of divalent cations (Figure 6).

Discussion

The main finding of this study is that [^{35}S]-ATPγS, in the absence of divalent cations, can label a population of high affinity sites in rat vas deferens, which appear to be the same as the sites labelled by [^{3}H]-αβmeATP, and whose binding characterics suggest that they may be P_{2X} purinoceptors.

A detailed characterization of the [^{35}S]-ATPγS binding sites in rat vas deferens could only be performed in the absence of divalent cations, since, although the binding of [^{35}S]-ATPγS was markedly increased by calcium ions, the binding characteristics of the [^{35}S]-ATPγS sites labelled in the presence of calcium were complex. Thus, biphasic competition curves were obtained for all agonists suggesting that the radioligand was labelling at least two populations of specific binding site. The most notable feature of the [^{35}S]-ATPγS binding sites labelled in the presence of calcium was their high affinity for GTPγS, which inhibited approximately 75% of binding with high affinity (pIC_{50} = 7.5). This relatively high affinity of GTPγS was similar to its potency in inhibiting the binding of [^{35}S]-ADPβS to P_{2Y} purinoceptors in turkey erythrocyte membranes (Cooper *et al.*, 1989) and so may suggest that a major component of [^{35}S]-ATPγS binding in the presence of calcium could be to a G-protein coupled purinoceptor. Since ATPγS is also an agonist at both the P_{2Y} and P_{2U} purinoceptors, it is possible that the sites may correspond to one of these receptors, although the relatively low affinity of UTP makes the possibility that the sites are P_{2U} purinoceptors unlikely. Irrespective of the nature of the [^{35}S]-ATPγS sites with high affinity for GTPγS, it is unlikely that they represent P_{2X} purinoceptors since the P_{2X} purinoceptor is defined, structurally, as a ligand-gated cation channel (Fredholm *et al.*, 1994) and so should not be affected by guanine nucleotides.

Due to the heterogeneity of the [^{35}S]-ATPγS binding sites when studied in the presence of calcium, an attempt was made to occlude those sites with high affinity for GTPγS. However, even in the presence of 1 μM GTPγS the heterogeneity of [^{35}S]-ATPγS binding sites was still evident. A further complication of the studies performed in the presence of calcium was the unexpected finding that the membranes could metabolize ATP under these conditions even though assays were conducted at 4°C. It was therefore not considered worthwhile to further study the binding of [^{35}S]-ATPγS in the presence of calcium.

When calcium ions were omitted and EDTA included in the assays, not only was metabolism of ATP undetectable but [^{35}S]-ATPγS bound in a reversible manner to a single population of high affinity binding sites. The capacity of these sites (4.27 pmol mg^{-1} protein) was similar to previous estimates for the density of high affinity [^{3}H]-αβmeATP binding sites in this tissue (1.01–18.4 pmol mg^{-1} protein; Michel & Humphrey, 1993; 1994). In competition studies, performed in the absence of divalent cations, a number of P_2 purinoceptor agonists and antagonists competed for a single population of [^{35}S]-ATPγS binding sites, providing additional evidence that the radioligand was labelling a single population of high affinity sites. These [^{35}S]-ATPγS binding sites displayed a high degree of specificity for P_2 purinoceptor ligands since binding was not inhibited by a number of adenosine receptor ligands, NBTI, staurosporine, thiosulphite and a number of ATPase inhibitors.

A worthwhile comparison of the binding sites labelled by [^{35}S]-ATPγS in rat vas deferens in the absence of divalent cations with the [^{3}H]-αβmeATP binding sites previously characterized in this tissue (Michel & Humphrey, 1993) was complicated because previous studies with [^{3}H]-αβmeATP had been performed in the presence of calcium. Calcium is known to affect the interaction of nucleotides with their receptors in at least two ways. First, it is apparent that the chelation state of ATP is important for its potency as a P_{2X} purinoceptor agonist and it has been suggested that ATP^{4-}, which is the predominant form of ATP in the absence of divalent cations, is the preferred agonist when compared to $Mg\text{-}ATP^{2-}$ or $Ca\text{-}ATP^{2-}$ which predominate in the presence of divalent cations

(Fine *et al.*, 1989; Fedan *et al.*, 1990; Lustig *et al.*, 1992; Motte *et al.*, 1993). Hence, the presence of calcium ions could reduce affinity estimates for those agonists which can adopt the tetrabasic configuration. Secondly, the metabolism of hydrolysable analogues of ATP is dramatically affected by calcium (Khakh *et al.*, 1995b) and it is possible that the hydrolysable nucleotides would be metabolized in its presence. Indeed we have shown that, even at 4°C, significant metabolism of low concentrations of ATP occurs.

Given these complexities it is not surprising that affinity estimates for a number of nucleotide analogues at the [^{3}H]-αβmeATP and [^{35}S]-ATPγS binding sites were very different. Affinity estimates for ATP, 2-meS-ATP, ATPγS and ADP at the [^{35}S]-ATPγS binding sites (pIC_{50} values of 9.01, 8.79, 8.73 and 7.24, respectively) were higher than their respective pIC_{50} values of 6.30, 7.38, 6.53 and 6.11 at the [^{3}H]-αβmeATP binding sites (Michel & Humphrey, 1993). In contrast, affinity estimates for αβmeATP and L-βγmeATP (pIC_{50} values of 7.57 and 5.83) at the [^{35}S]-ATPγS binding sites were less than the respective pIC_{50} values of 8.25 (Michel & Humphrey, 1993) and 7.7 (Michel *et al.*, 1994) at the [^{3}H]-αβmeATP binding sites.

The higher affinity estimates of ATP, ATPγS and 2-meS-ATP determined in the present studies on the [^{35}S]-ATPγS binding sites compared to previous affinity estimates determined at the [^{3}H]-αβmeATP binding sites could have been due to the effect of calcium on both the ionisation state and metabolism of the nucleotides in the later studies. Thus, 2-meS-ATP, like ATP possesses the unmodified triphosphate side chain required for formation of ATP^{4-}, while in ATPγS the γ-thio substitution in the triphosphate chain decreases its pK_a, relative to ATP, so favouring formation of the ATP^{4-} structure (Yount, 1975). For these agonists, at pH 7.4, a significant proportion of the compound would exist in the ATP^{4-} conformation in the absence of divalent cations, but in the presence of calcium a large proportion of this ATP^{4-} would be chelated as $Ca\text{-}ATP^{2-}$. Thus, for ATP it has been calculated that in physiological medium containing 1 mM $CaCl_2$, about 2.4% of the ATP is present as ATP^{4-}, but in the absence of divalent cations 48% of ATP is in the ATP^{4-} form (Motte *et al.*, 1993). This could account for about a 20 fold greater affinity in studies performed in the absence than in the presence of calcium ions. With respect to metabolism it is well documented that ATP and 2-meS-ATP are subject to extensive metabolism (Welford *et al.*, 1986; 1987). Furthermore, we have demonstrated metabolism of ATP at 4°C in this study, while a calcium-dependent metabolism of both ATP and 2-meS-ATP has been demonstrated by Kharkh *et al.* (1995b). Although ATPγS is often described as being hydrolysis-resistant it can serve as a substrate for several enzymes (Yount, 1975) and has been shown to be metabolized in skeletal muscle (Cascalheira & Sebastião, 1992).

The higher affinity of αβmeATP and L-βγmeATP for the [^{3}H]-αβmeATP binding sites than for [^{35}S]-ATPγS binding sites is more difficult to explain. Metabolism of αβmeATP and L-βγmeATP in the presence or absence of calcium ions at 4°C seems unlikely. Even at 37°C, metabolism of αβmeATP and βγmeATP was not detectable in guinea-pig bladder (Welford *et al.*, 1987) and taenia coli (Welford *et al.*, 1986) and was almost insignificant in guinea-pig vas deferens when compared to ATP (Ziganshin *et al.*, 1994). In our studies in vas deferens we could detect no metabolism of αβmeATP under conditions where rapid metabolism of ATP and 2-meS-ATP occurred (Khakh *et al.*, 1995b). To our knowledge, the ionisation constants for αβmeATP and L-βγmeATP have not been described nor is it known if these compounds are active as their $\alpha\beta meATP^{4-}$ and L-$\beta\gamma meATP^{4-}$ forms, respectively, in functional studies on the P_{2X} purinoceptor. We can therefore only note that there is an important role of calcium in modulating the high affinity binding of αβmeATP and L-βγmeATP to their binding sites/receptors, either by virtue of the calcium-chelated forms possessing higher affinity or through a direct affect of calcium on the binding site.

While it is clearly difficult to compare the [^{35}S]-ATPγS and [^{3}H]-αβmeATP binding sites when studied under their individual optimal conditions we have characterized the binding of [^{3}H]-αβmeATP in the absence of calcium ions and, on the basis of these studies, we suggest that [^{3}H]-αβmeATP and [^{35}S]-ATPγS are probably labelling the same sites. Thus, in the absence of divalent cations, saturation studies with [^{3}H]-αβmeATP reveal that it binds with relatively low affinity to a single population of sites and that the pK_D value of 7.76 (Michel & Humphrey, 1994) is very similar to the pIC_{50} value of 7.87 for αβmeATP at the [^{35}S]-ATPγS binding sites. Furthermore, affinity estimates for the 8 nucleotide analogues at the [^{3}H]-αβmeATP binding sites in the absence of divalent cations were almost identical to those determined in studies on the [^{35}S]-ATPγS binding sites in the absence of calcium ions.

In contrast to the agonist affinity estimates, the affinity estimates for the P_{2X} purinoceptor antagonists were not markedly affected by calcium. Thus, the pIC_{50} values for suramin, cibacron blue, PPADS, pyridoxal 5-phosphate and DIDS of 5.20, 5.70, 4.23, 3.42 and 4.75, respectively, determined at the [^{35}S]-ATPγS binding sites were not greatly different to their respective pIC_{50} values of 5.15, 5.34, 5.07, 3.69 and 4.50 determined at the [^{3}H]-αβmeATP binding sites (Khakh *et al.*, 1994; Trezise *et al.*, 1994 and unpublished observations). While this provides some additional evidence that the [^{3}H]-αβmeATP and [^{35}S]-ATPγS binding sites are the same, it is necessary to stress the limited specificity of the P_{2X} purinoceptor antagonists used. In particular, when the antagonists were studied as inhibitors of Ca-ATPase in vas deferenes membranes, the pIC_{50} values of suramin, cibacron blue, PPADS and pyridoxal 5-phosphate of 5.4, 5.9, 4.4 and 3.2, respectively, for inhibiting this enzyme activity (Khakh *et al.*, 1995a) were similar to their affinity estimates for the [^{35}S]-ATPγS binding sites. This latter observation serves to highlight the need for improved and more specific antagonists of the P_{2X} purinoceptor.

Until recently, the classification of P_{2X} purinoceptors has relied upon the rank order of agonist potencies and on the basis of the current classification of P_{2X} purinoceptors as displaying the rank order of potency of αβmeATP > 2-meS-ATP (Burnstock & Kennedy, 1985), it would be difficult to characterize the [^{35}S]-ATPγS binding sites as P_{2X} purinoceptors. However, given the potential complications of metabolism in studies using hydrolysable nucleotides, as revealed in studies using ecto-ATPase inhibitors (Crack *et al.*, 1994; 1995), it is now likely that the rank order of potency as determined in the majority of functional studies is no longer appropriate. Instead, the rank order of potency of ATP ≥ 2-meS-ATP ≥ αβmeATP, as determined in rat blood vessels (Evans & Kennedy, 1994) and rat vas deferens (Khakh *et al.*, 1995b), using isolated cells and concentration-clamp perfusion to exclude the complications of metabolism, is more appropriate. This rank order of potency is similar to that obtained on the [^{35}S]-ATPγS binding sites. While the potency of αβmeATP was clearly lower than that of ATP and 2-meS-ATP in the binding studies this discrepancy could reflect the differential effects of calcium ions, present in functional studies, on the affinity estimates for αβmeATP, ATP and ATPγS. In this respect the rank order of potency of ATP ≥ 2-meSATP ≥ ATPγS > αβmeATP was almost identical to that found in studies on the P_{2X} purinoceptor mediating depolarization of rat vagus nerve (Trezise *et al.*, 1993). The study by Tresize and colleagues, like the present one, was performed in the absence of divalent cations and is perhaps therefore the most appropriate for comparative purposes. However, the caveat that recent cloning (Valera *et al.*, 1994; Brake *et al.*, 1994) and functional (Michel *et al.*, 1994) studies have indicated differences between smooth muscle and neuronal P_{2X} purinoceptors should be acknowledged.

We have provided evidence that [^{35}S]-ATPγS exhibits high affinity binding to sites which appear to represent labelling of P_{2X} purinoceptors. Although [^{3}H]-αβmeATP is also a useful radioligand, the recent cloning of the P_{2X} purinoceptor from PC12 cells (Brake *et al.*, 1994) indicates that αβmeATP-insensitive, but ATPγS-sensitive, subtypes of P_{2X} purinoceptor

exist and therefore [^{35}S]-ATPγS may be a more useful radioligand for studying these receptors. Furthermore, its high specific activity offers a major advantage where receptor density is low. With the caveats necessary for accepting that [^{35}S]-ATPγS labels the P_{2X} purinoceptor, definitive proof that it binds to P_{2X} purinoceptors will only be obtained when binding to recombinant P_{2X} purinoceptors can be demonstrated. In this respect we have recently been able to demonstrate that transfection of CHO-K1 cells with a human bladder P_{2X} purinoceptor results in the appearance of [^{35}S]-ATPγS binding sites, and that affinity estimates for the nucleotide analogues used in the present study for these sites are similar to the affinity estimates obtained in vas deferens (G. Buell, K. Lundstrom and A.D. Michel, unpublished observation).

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