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Characterization of [^{35}S]-ATPαS and [^{3}H]-α,β-MeATP binding sites in rat brain cortical synaptosomes: regulation of ligand binding by divalent cations

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1 We made a comparative analysis of the binding characteristics of the radioligands [^{35}S]-ATPαS and [^{3}H]-α,β-MeATP in order to test whether these ligands can be used to analyse P_2-purinoceptors in synaptosomal membranes from rat brain cortex.

2 Synaptosomes possess sites with high affinity for [^{35}S]-ATPαS ($K_d = 22.2 \pm 9.1$ nM, $B_{max} = 14.8$ pmol mg^{-1} protein). The rank order of the competition potency of the different compounds (ATPαS, ATP, ATPγS > ADPβS, 2-MeSATP > deoxyATP, ADP > > UTP, α,β-MeATP, AMP, Reactive Blue-2, suramin, isoPPADS) is consistent with pharmacological properties of P_{2Y}-purinoceptors.

3 Under identical conditions [^{35}S]-ATPαS and [^{3}H]-α,β-MeATP bind to different binding sites at synaptosomal membranes from rat brain cortex. The affinity of the [^{3}H]-α,β-MeATP binding sites ($K_d = 13.7 \pm 1.8$ nM, $B_{max} = 6.34 \pm 0.28$ pmol mg^{-1} protein) was 38 fold higher than the potency of α,β-MeATP to displace [^{35}S]-ATPαS binding ($K_i = 0.52$ μM). ATP and ADPβS competed at both binding sites with different affinities, 60 fold and 175 fold, respectively. The other agonists tested (2-MeSATP, UTP, GTP) did not affect specific [^{35}H]-α,β-MeATP binding at concentrations up to 100 μM. The antagonists (suramin, isoPPADS, Evan's Blue) showed completely different affinities for both binding sites.

4 Binding of [^{35}S]-ATPαS on synaptosomes was regulated by GTP, which is indicative for G-protein coupled receptors. The K_d value for the high affinity binding site was reduced in the presence of GTP about 5 fold (from 1.8 nM to 8.6 nM). In the presence of Mg^{2+} the affinity was increased (K_d 1.8 nM versus 22 nM in the absence of Mg^{2+}).

5 The binding of both radioligands was regulated in an opposite manner by physiological concentrations of Ca^{2+} and Mg^{2+}. Binding of [^{3}H]-α,β-MeATP to synaptosomal membranes was increased 3 fold by raising the Ca^{2+} concentration from 10 μM to 1 mM, whereas the addition of Mg^{2+} in the same concentration range resulted in an 80% reduction of the binding. In contrast, [^{35}S]-ATPαS binding was not influenced at the same range of Ca^{2+} or Mg^{2+} concentrations (10 μM to 1 mM). The addition of Mg^{2+} (5 mM) increased the affinity of [^{35}S]-ATPαS for the high affinity site 10 fold.

6 Diadenosine polyphosphates had a bimodal effect on [^{35}S]-ATPαS binding to synaptosomal membranes. AP_5A and Ap_6A enhanced binding of [^{35}S]-ATPαS 1.6 fold in a concentration range between 0.1 and 50 μM. Ap_3A was a weak inhibitor with a K_i value of 7.2 μM. Ap_4A, AP_5A and Ap_6A inhibited with K_i values > 100 μM. These data support the concept that diadenosine polyphosphates do not directly interact with ATPαS binding sites.

7 In conclusion, on the basis of present knowledge of the interaction of P_2-purinoceptor active compounds with P_{2X}- and/or P_{2Y}-purinoceptors, our data strongly suggest that [^{35}S]-ATPαS is a useful tool to study P_{2Y}-purinoceptors. Thus, the [^{35}S]-ATPαS binding site might to a large extent represent P_{2Y}-purinoceptors in synaptosomes from rat brain cortex. The nucleotide binding is regulated by G proteins, indicated by the effects of GTP/Mg^{2+} on binding.

Keywords: Purinoceptors; synaptosomes; diadenosine polyphosphates; P_{2Y}-purinoceptors; P_{2X}-purinoceptors

Introduction

Extracellular nucleotides elicit physiological responses in neuronal cells like in other cell types through receptors at the plasma membrane, which are termed P_2-purinoceptors (Burnstock *et al*., 1990; Zimmermann, 1994; Harden *et al*., 1995). The signal transduction mechanisms coupled to the P_2-purinoceptors serve to distinguish two major classes of P_2-purinoceptors. The ionotropic P_{2X}-purinoceptors, which are a unique class of ligand-gated ion channels, and the metabotropic, G protein-coupled P_{2Y}-purinoceptors (including the P_{2U}-purinoceptor) which interact either with adenylate cyclase or phospholipase C. Several isoforms of the P_{2X}-purinoceptor (Valera *et al*., 1994; Brake *et al*., 1994; Bo *et al*., 1995; Buell *et al*., 1996; Soto *et al*., 1996) and P_{2Y}-purinoceptor (Webb *et al*., 1993; Parr *et al*., 1994; Filtz *et al*., 1994) have been identified. Recently another G protein-coupled P_2-purinoceptor, termed P_{2D}, which is specifically activated by diadenosine polyphosphates has been postulated to be present also in the central nervous system (Pintor & Miras-Portugal, 1995).

Characterization of the functional properties of P_2-purinoceptors in the CNS revealed that in the physiological processes at synapses P_{2X}-purinoceptors (Evans *et al*., 1992; Edwards *et al*., 1992; Cloues, 1995; Sun & Stanley, 1996) as well as P_{2Y}-purinoceptors (Krishtal *et al*., 1988; Illes & Nörenberg, 1993; von Kügelgen *et al*., 1994) are involved. Furthermore, the distribution and the binding characteristics of the P_{2X}-purinoceptors were investigated by binding studies and autoradiography with [^{3}H]-α,β-methylene adenosine 5′-triphosphate ([^{3}H]-α,β-MeATP; Bo & Burnstock, 1994; Balcar *et al*., 1995; Mockett *et al*., 1995; Michel & Humphrey, 1994) and thiolabelled ATP analogues. [^{35}S]-2′-deoxyadenosine 5′-O-(1-thiotriphosphate) ([^{35}S]-deoxyATPαS), [^{35}S]-ATPαS and [^{35}S]-ATPγS have been used to carry out binding studies to characterize metabotropic P_2-purinoceptors in brain and other tissues (Keppens *et al*., 1989; Pintor *et al*., 1993; Simon *et al*., 1995; Michel & Humphrey, 1996).

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Evidence for the occurrence of G protein-coupled P_2-purinoceptors in brain has been obtained from expression experiments by injection of total brain mRNA into *Xenopus* oocytes or from the pharmacological studies of nucleotide effects in astrocytes (Fournier *et al*., 1990; Bruner & Murphy, 1990). In addition, P_2-purinoceptors induce nitric oxide-dependent cyclic GMP production in the neuronal cell line NG 108-15 (Reiser, 1995) or evoke Ca^{2+}-oscillations associated with Ca^{2+}-influx in rat glioma cells (Czubayko & Reiser, 1996a,b). Moreover, P_{2Y}-like P_2-purinoceptors have been shown to mediate a decreased noradrenaline release on postganglionic sympathetic neurones (von Kügelgen *et al*., 1994).

However, little is known so far about the abundance and ligand binding characteristics of the metabotropic P_{2Y}-purinoceptors at synapses in the CNS. Synaptosomes are the most suitable preparation for studying these receptors. We therefore examined the binding characteristics of the radioligand [^{35}S]-ATPαS to assess whether it can be used to study metabotropic, G protein-coupled P_2-purinoceptors in synaptosomal membranes of rat brain cortex. The results are juxtaposed on the data obtained with [^{3}H]-α,β-MeATP binding, in order possibly to characterize P_{2X}-purinoceptors and to elucidate the binding selectivity of the two radioligands.

We show here that synaptosomes from rat brain cortex possess high and low affinity binding sites for [^{35}S]-ATPαS. The high affinity [^{35}S]-ATPαS binding sites at least partly reflect P_{2Y}-purinoceptors based on the affinity profile of the different compounds active at P_2-purinoceptors. Moreover, there was a clear difference between the binding characteristics found with [^{35}S]-ATPαS and with [^{3}H]-α,β-MeATP. The binding of the two radioligands was regulated in an opposite way by Ca^{2+} and Mg^{2+}. The regulation of [^{35}S]-ATPαS binding by guanosine 5′-triphosphate (GTP) was characteristic of G protein-coupled receptors. Furthermore, diadenosine polyphosphates, which are also found in brain synaptosomes were weak competitors at ATPαS binding sites.

Methods

Preparation of synaptosomal membranes

The synaptosomal membranes were prepared from synaptosomes isolated as described by Gordon-Weeks (1987), by sucrose density gradient centrifugation. Cortices were dissected from whole rat brain, trimmed free of white matter and homogenized in 15 volumes of 0.32 M sucrose, 10 mM HEPES-NaOH, pH 7.4 (buffer A) by 10 strokes with a motor-driven teflon pestle (800 r.p.m.) in an Elvehjiem potter at 4°C. Nuclei and cellular debris were pelleted by centrifugation at 1000 g_{max} for 5 min and the supernatant was then centrifuged at 12,000 $\times g_{max}$ for 20 min. The resulting pellet was washed twice, resuspended in buffer A and layered on a sucrose step gradient (1.2, 1.0 and 0.8 M sucrose in buffer A). The gradients were centrifuged at 86,000 $\times g_{av}$ for 120 min, the synaptosomes collected from the interface of the 1.0/1.2 M sucrose gradient steps and diluted with buffer B (5 mM Tris.HCl, pH 8.1, 50 μM $CaCl_2$) to lyze the synaptosomes. The synaptosomal membranes were isolated by centrifuging the lyzed synaptosomes on a second sucrose gradient (1.2 M, 1.0 and 0.85 M sucrose in buffer B) at 63.600 $\times g_{max}$ for 105 min. The synaptosomal membranes were removed at the interface of the 1.0/1.2 M sucrose gradient steps, pelleted by centrifugation at 150,000 $\times g_{max}$ for 30 min, washed with buffer A containing 1 mM EDTA, resuspended in buffer A and stored at −80°C. Protein was determined by the Lowry method with BSA as standard.

Binding experiments

In saturation binding experiments of [^{35}S]-ATPαS or [^{3}H]-α,β-MeATP total binding was measured by incubating synaptosomal membranes (25 μg of protein) for 35 min at 4°C in an incubation medium containing 25 mM HEPES, pH 7.4, 50 mM NaCl, 5 mM KCl (binding buffer) with increasing concentrations of [^{35}S]-ATPαS (0.1 – 100 nM) or [^{3}H]-α,β-MeATP (0.3 – 200 nM), respectively in a final assay volume of 100 μl. In competition binding experiments binding of 1 nM [^{35}S]-ATPαS, 1 nM [^{35}S]-UTPαS or 3 nM [^{3}H]-α,β-MeATP was determined in binding buffer under the same conditions used for saturation studies in the presence of various unlabelled agonists and antagonists in a concentration range from 1 nM to 1 mM. The effect of GTP or GTPγS in the presence of 5 mM $MgCl_2$ and of α,β-MeATP or Evan's Blue on [^{35}S]-ATPαS binding was measured by preincubation of the synaptosomal membranes for 30 min at 4°C with these compounds. Equilibrium binding was achieved for all radioligands used after about 30 min and binding remained constant for at least another two hours. The incubation was terminated by pelleting the membranes by centrifugation at 25,000 $\times g$ for 12 min. The pellet was rinsed twice with 150 μl of icecold binding buffer and bound radioligand was measured in the pellet after solubilization with 100 μl 1.5% (w/v) sodium dodecyl sulphate (SDS) solution using a liquid scintillation β-counter (Canberra Packard). In saturation and competition studies nonspecific binding was determined in the presence of 100 μM ATPαS or 100 μM α,β-MeATP and was between 5 – 15% of total binding. Total binding never exceeded 15% of the ligand concentration used.

Materials

ATP, ADP, AMP, ATPγS, adenosine 5′-O-(2-thiodiphosphate) (ADPβS), α,β-MeATP, deoxyATP, Reactive blue-2, GTP, uridine 5′-triphosphate (UTP), Evan's Blue (P-1,P-3-di(adenosine 5′) triphosphate (Ap_3A) and P-1,P-6-di(adenosine 5′) hexaphosphate (Ap_6A) were from Sigma (Deisenhofen, Germany). The diadenosine polyphosphates Ap_4A and Ap_5A were a gift from Dr H. Schlüter, (Ruhr-University Bochum, Germany). ATPαS was purchased from Calbiochem (Bad Soden, Germany). GTPγS was bought from Biomol (Hamburg, Germany). Radiolabelled [^{35}S]-ATPαS and [^{35}S]-UTPαS were purchased from DuPont NEN (Bad Homburg, Germany) and [^{3}H]-α,β-MeATP (26 TBq mmol^{-1}) was from Amersham (Braunschweig, Germany). Suramin was a gift from Boehringer Mannheim. Pyridoxal-phosphate-6-azo phenyl-2′,4′-disulphonic acid (IsoPPADS) and 2-methylthio adenosine 5′-triphosphate (2-MeSATP) were from Biotrend (Köln, Germany). Pyridoxalphosphate was purchased from Serva (Heidelberg, Germany) and all other chemicals were of the highest purity available either from Merck (Darmstadt, Germany) or Serva (Heidelberg, Germany).

Data analysis

The binding data of saturation and competition binding were analysed by nonlinear regression with models using one or two binding sites by the RADLIG programme ((RADLIG program, Biosoft Corp.). The K_d and K_i values were tested for significant difference ($P<0.05$) with the RADLIG programme by using Scatchard analysis.

Results

The synaptosomal membranes obtained from rat brain cortex were examined whether they contained high affinity binding sites for [^{35}S]-ATPαS or [^{3}H]-α,β-MeATP. The analysis of the binding data of [^{35}S]-ATPαS at a concentration range from 0.1 to 100 nM by nonlinear regression in models with one or two binding sites clearly revealed the existence of two distinct binding sites (Figure 1a, b). The high affinity binding site displayed a K_{d1} of 22.2 ± 9.1 nM ($n=5$) with a maximal binding capacity of B_{max} of 14.8 ± 2.3 pmol mg^{-1} protein. [^{35}S]-ATPαS bound to the low affinity binding site with a K_{d2} of 2.6 ± 1.1 μM and B_{max} of 426 ± 62 pmol mg^{-1} protein. When specific binding of [^{35}S]-ATPαS was examined by displacement of binding

Table 1 Competition of binding of 1.0 nM [^{35}S]-ATPαS to synaptosomal membranes (rat brain cortex) by various agonists and antagonists

Ligand	K_i-*value* (nM) *High affinity site*	*Low affinity site*
ATPαS	21±5 (n=7)	4,100±545
ATP	29±5 (n=5)	4,920±415
ATPγS	38±4 (n=3)	5,500±265
ADPβS	52 (n=2)	7,090
2-MeSATP	64±7 (n=3)	5,160±380
deoxyATP	107 (n=2)	7,230
ADP	123 (n=2)	8,010
AMP	351 (n=2)	8,645
UTP	435±167 (n=3)	7,640±115
α,β-MeATP	518±119 (n=4)	7,980±220
Reactive Blue-2	380±204 (n=3)	7,255±135
Suramin (Germanin)	284±159 (n=3)	5,145±215
IsoPPADS	371±258 (n=3)	10,355±430
Pyridoxalphosphate	835±195 (n=3)	735,000
Evan's Blue	6450 (n=2)	87,160

The different compounds active at P_2-purinoceptors were incubated with synaptosomal membranes (25 μg of protein) for 35–40 min in a concentration range 1 nM to 1 mM. The K_i value for each ligand was determined by the RADLIG programme with K_d values of 22.2 nM (high affinity) and 2.6 μM (low affinity) for [^{35}S]-ATPαS. All values are expressed as mean values (n=2) or mean values±s.e.mean from three to seven experiments performed in duplicate with three different preparations. n=number of experiments.

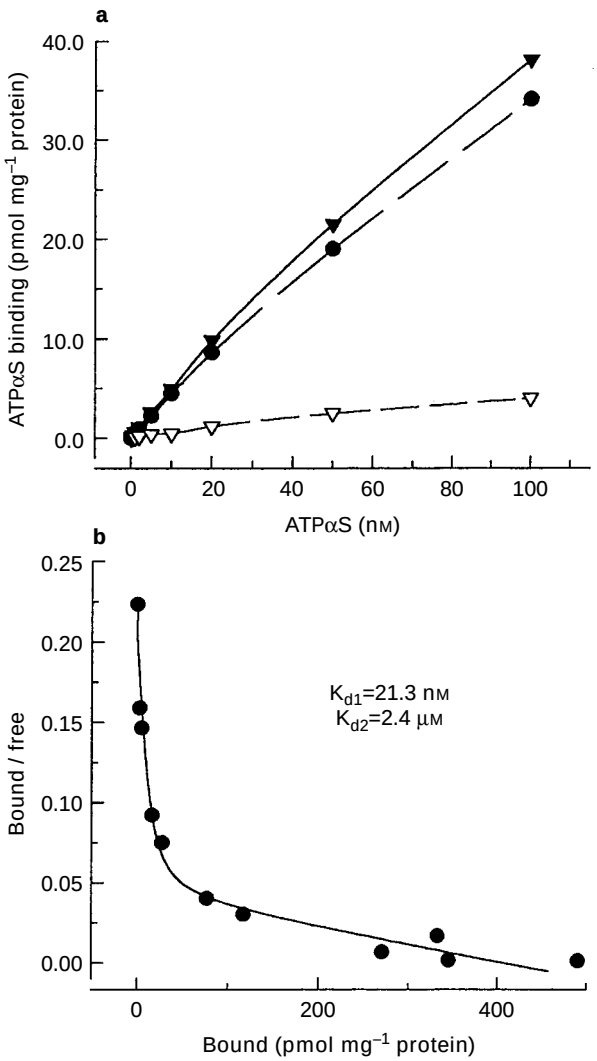

Figure 1 Binding of [^{35}S]-ATPαS to synaptosomal membranes. (a) Total binding (▼) was measured as described in Methods for 35 min at 4°C with increasing concentrations of [^{35}S]-ATPαS (0.1–100 nM) in duplicate. Nonspecific binding (▽) was measured in the presence of 100 μM ATPαS. The difference yielded specific binding (●). (b) Scatchard plot of the data in (a) analysed by nonlinear regression with the curve displayed giving the best fit to the data (r^2=0.998). The experiment shown is typical of 5 experiments with 3 different membrane preparations.

by unlabelled ATPαS, the analysis of the data also revealed two binding sites with K_i values of 21±5 nM (n=7) and 4.1±0.55 μM (n=7) (Table 1), which were in good agreement with the K_d-values obtained in saturation experiments (see Figure 1). The low affinity sites (μM range) in the saturation experiments were derived from the computational fitting of the experimental data at the various ligand concentrations up to 200 nM (see Figure 1). Low affinity binding sites were also derived from the data of the displacement experiments with unlabelled ATPαS when the data were analysed by the computer programme under the assumption of cold saturation. Thus, these results support the two-site binding model as deduced from the saturation experiments.

The [^{35}S]-ATPαS binding sites were characterized by using agonists and antagonists active at P_2-purinoceptors. Binding curves in Figure 2 and the data in Table 1 demonstrate that ATPαS, ATPγS and ATP have the highest affinity for [^{35}S]-ATPαS binding sites. They displaced binding of 1 nM [^{35}S]-ATPαS to synaptosomal membranes with equal potency, followed by ADPβS, 2-MeSATP and deoxyATP, whereas α,β-MeATP was much less potent (Figure 2 and Table 1). The rank order of the affinity of various P_2-purinoceptor-active ligands (ATPαS, ATP, ATPγS > ADPβS, 2-MeSATP > deoxyATP, ADP >> UTP, α,β-MeATP, AMP) shown in Table 1 indicates binding of [^{35}S]-ATPαS to a high affinity binding site at a P_{2Y}-purinoceptor-like protein. The small reduction of specific [^{35}S-ATPαS binding by α,β-MeATP at concentrations between 0.1 and 10 μM (Figure 2), which might suggest that [^{35}S]-ATPαS also binds to P_{2X}-purinoceptors was not found consistently in the various experiments. Moreover, binding of ATPαS to α,β-MeATP-sensitive P_{2X}-purinoceptors is excluded by (i) analysis of [^{3}H]-α,β-MeATP binding (see below Figure 3 and Table 2), (ii) the finding that displacement of 1 nM [^{35}S]-ATPαS by ATPαS was not affected in experiments carried out in the presence of 0.5 μM α,β-MeATP (Table 3) and (iii) Evan's Blue, which had been found to interact preferentially with ecto-ATPases or P_{2X}-purinoceptors affected [^{3}H]-α,β-MeATP binding, but not [^{35}S]-ATPαS binding (see Figure 8).

GTP did not displace [^{35}S]-ATPαS binding at concentrations below 100 μM (Figure 2) and like α,β-MeATP and UTP, only partially displaced binding of [^{35}S]-ATPαS even at concentrations up to 1 mM. The P_2-purinoceptor antagonists Reactive Blue-2, suramin, isoPPADS, Evan's Blue and pyridoxalphosphate were also tested. Suramine, isoPPADS and Reactive Blue-2 were equally potent in inhibiting [^{35}S]-ATPαS binding to synaptosomal membranes (K_i values about 300 nM), whereas pyridoxalphosphate and Evan's Blue were about 2.5 fold and 20 fold less potent in competing for high affinity [^{35}S]-ATPαS binding (Table 1).

Binding of [^{35}S]-ATPαS to the low affinity site was suppressed with similar potency by all the agonists and antagonists tested (Table 1). This indicates that the low affinity binding site cannot be characterized by the different compounds applied. It should be noted that at the low affinity site the precision of the determination of the affinities of the various P_2-purinoceptor-active compounds is limited. This is due to the fact that under the experimental conditions used only 20% of the low affinity sites were occupied with radioligand.

It has been shown recently that high affinity [^{3}H]-α,β-MeATP sites at P_{2X}-purinoceptors in rat vas deferens membranes can be labelled with [^{35}S]-ATPγS (Michel & Humphrey, 1996). We therefore used [^{3}H]-α,β-MeATP to see whether in

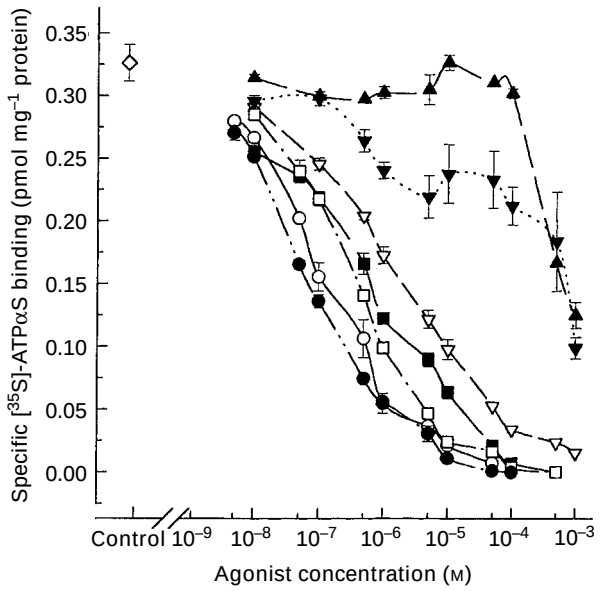

Figure 2 Competition of [^{35}S]-ATPαS binding to synaptosomal membranes by different P_2-purinoceptor active compounds and GTP. The incubation sample contained [^{35}S]-ATPαS (1 nM) and the various ligands at the concentrations indicated (1 nM – 1 mM). The data shown from one experiment performed in duplicate are typical for at least three independent experiments with three different membrane preparations. The ligands used were ATPαS (●), ATP (○), 2-MeSATP (□), ADPβS (■), 2-dATP (▽), α,β-MeATP (▼) and GTP (▲). Vertical lines show s.e.mean.

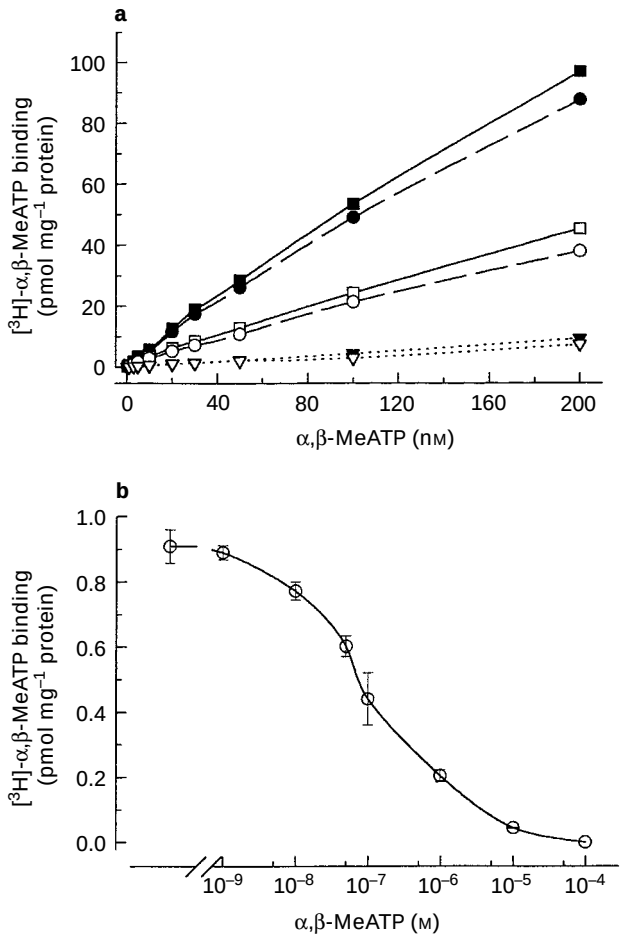

Figure 3 Binding of [^{3}H]-α,β-MeATP to synaptosomal membranes from cortex. (a) Total binding (■, □) was measured as described in Methods for 35 min at 4°C with increasing concentrations of [^{3}H]-α,β-MeATP (0.3 – 200 nM) in the presence (filled symbols ■, ●, ▼) or absence (open symbols (□, ○, ▽) of 0.5 mM Ca^{2+}, determined in duplicate. Nonspecific binding (▼, ▽) was measured in the presence of 100 μM α,β-MeATP. Specific binding is given by (●, ○). The experiment shown carried out in duplicate is typical for 5 experiments from 3 different membrane preparations. (b) Competition of 3 nM [^{3}H]-α,β-MeATP binding to synaptosomal membranes by increasing concentrations of α,β-MeATP. The data shown are representative for 5 experiments carried out in triplicate using four different membrane preparations. Vertical lines show s.e.mean.

synaptosomal membranes this radioligand will also bind to the sites detected with [^{35}S]-ATPαS. The analysis of the saturation binding studies of [^{3}H]-α,β-MeATP to synaptosomal membranes revealed only one high affinity binding site with $K_d = 13.7 \pm 1.8$ nM ($n = 5$) and a maximal binding capacity $B_{max} = 6.34 \pm 0.28$ pmol mg^{-1} protein (Figure 3a). Specific binding of [^{3}H]-α,β-MeATP was greatly increased in the presence of 0.5 mM Ca^{2+} (Figure 3a). There was no significant shift in the affinity of the binding sites ($K_d = 8.7 \pm 4.6$ nM; $n = 3$) but an increase in the maximal binding capacity ($B_{max} = 28.5 \pm 6.2$ pmol mg^{-1} protein). A detailed analysis of the influence of divalent cations on [^{3}H]-α,β-MeATP binding is presented in Figure 6. Analysis of the displacement curve for [^{3}H]-α,β-MeATP (Figure 3b) also revealed only one binding site ($K_d = 15.2 \pm 0.9$ nM; $n = 5$) with $B_{max} = 5.89 \pm 0.35$ pmol mg^{-1} protein. Although the binding values of [^{3}H]-α,β-MeATP in the experiment in Figure 3a did not reach saturation at a ligand concentration of 200 nM the very similar K_d and B_{max} values derived from the displacement experiments (Figure 3b) validate the results obtained from the saturation experiment.

We characterized the [^{3}H]-α,β-MeATP binding site by use of different agonists, which have been shown usually to act preferentially on P_{2Y}-purinoceptors, and by GTP (Table 2). The rank order for the displacement potency of the compounds tested was completely different from that found at the high affinity [^{35}S]-ATPαS binding site (for comparison see Table 1). The P_2-purinoceptor agonist α,β-MeATP was the most potent competing compound with $K_i = 19 \pm 5$ nM ($n = 3$) while ATP was less potent with a K_i value of 180 ± 40 nM ($n = 3$). ADPβS inhibited binding with an affinity in the micromolar range (0.91 ± 0.31 μM, $n = 3$). All other compounds tested (2-MeSATP, UTP, GTP) did not affect specific [^{3}H]-α,β-MeATP binding at concentrations of up to 100 μM.

The affinity values derived from the P_2-purinoceptor-active antagonists at the [^{3}H]-α,β-MeATP binding site were different from those at the high affinity [^{35}S]-ATPαS binding site. Suramin and isoPPADS competed with 2 to 3 fold lower potency. In contrast Evan's Blue showed a 6 fold higher affinity for the [^{3}H]-α,β-MeATP binding site than for the high affinity [^{35}S]-ATPαS binding site (Tables 1 and 2).

Table 2 Competition of binding of 3.0 nM [^{3}H]-α,β-MeATP to synaptosomal membranes (rat brain cortex) by various agonists and antagonists

Ligand	K_i *value* (nM)
α,β-MeATP	19 ± 5 ($n = 3$)
ATP	180 ± 40 ($n = 3$)
ADPβS	910 ± 310 ($n = 3$)
2-MeSATP	> 100 μM ($n = 2$)
UTP	> 100 μM ($n = 2$)
GTP	> 100 μM ($n = 2$)
Suramin	885 ($n = 2$)
Evan's Blue	1,060 ($n = 2$)
IsoPPADS	3,900 ($n = 2$)

The different compounds were incubated with synaptosomal membranes (25 μg of protein) for 35 – 40 min in a concentration range 1 nM to 100 μM. The K_i value for each ligand was determined by the RADLIG programme with K_d value of 13.7 nM for [^{3}H]-α,β-MeATP. All values are expressed as mean values ($n = 2$) or mean values ± s.e.mean from three experiments performed in triplicate with three different preparations. n = number of experiments.

Table 3 Effect of GTP, Mg^{2+} and α,β-MeATP on binding of 1.0 nM [^{35}S]-ATPαS to synaptosomal membranes (rat brain cortex)

	K_i-*value* (nM)	
Ligand	*High affinity site*	*Low affinity site*
ATPαS (no Mg^{2+}) ($n=7$)	21±5	4,100±545
GTP ($n=2$)	7160	415,000
GTPγS ($n=2$)	3780	93,500
ATPαS (+5 mM Mg^{2+}) ($n=3$)	1.75±0.31	675±183
ATPαS (+5 mM Mg^{2+}; 5 μM GTP) ($n=3$)	8.55±1.26	1,650±419
ATPαS (+0.5 μM a,β-MeATP ($n=3$)	17±4	3,280±410

For measurement of [^{35}S]-ATPαS binding in the presence of GTP or GTPγS, synaptosomal membranes (25 μg of protein) were preincubated with 5 mM Mg^{2+} and GTP/GTPγS for 30 min at 4°C. Then the test incubation was started by addition of [^{35}S]-ATPαS for a further 35 min. Values are expressed as mean values ($n=2$) or mean values±s.e.mean and refer to the determination of high and low affinity binding.

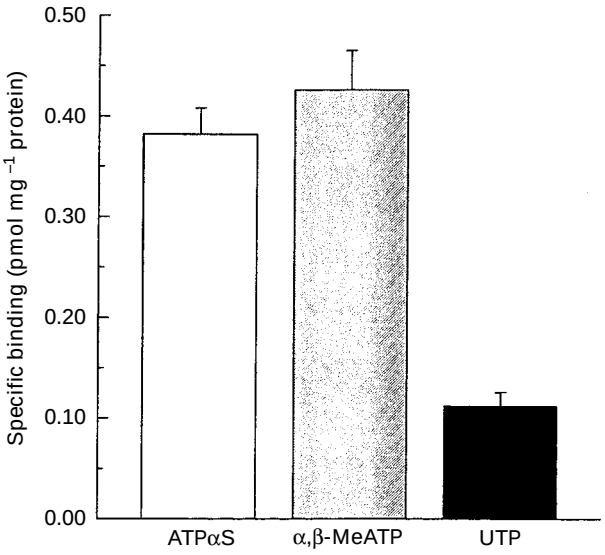

Figure 4 Determination of P_2-purinoceptor subtypes in synaptosomal membranes from cortex. Membranes (25 μg of protein) were assayed with 1 nM [^{35}S]-ATPαS, [^{3}H]-α,β-MeATP or [^{35}S]-UTP in the presence and absence of 100 μM ATPαS, α,β-MeATP or UTP, respectively. Columns represent the mean±s.e.mean of the specific binding of each ligand from three determinations.

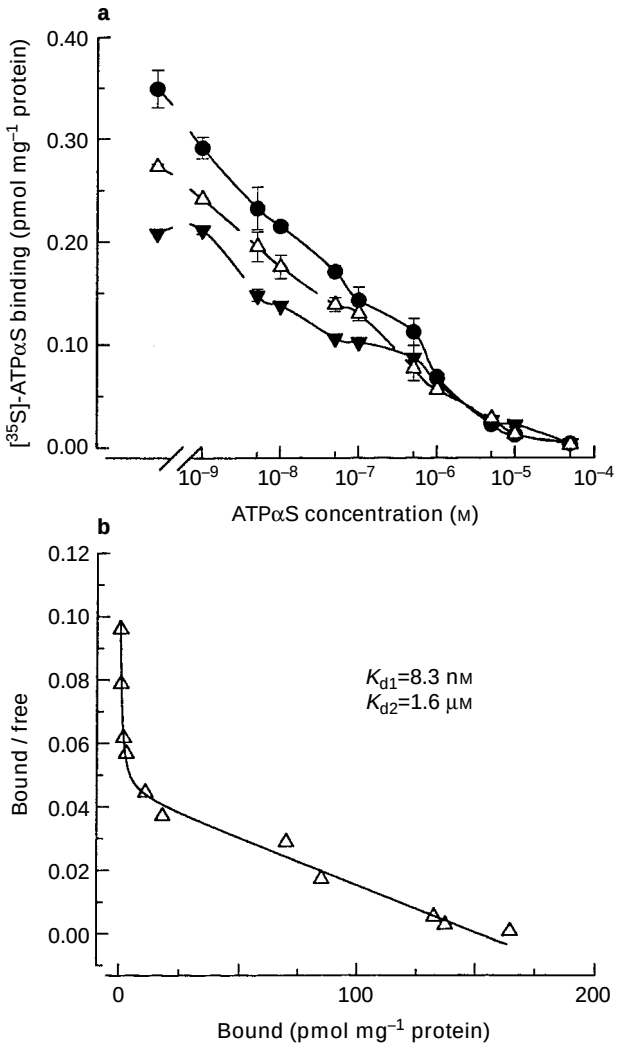

Figure 5 Guanine nucleotide inhibition of [^{35}S]-ATPαS binding to synaptosomes. Binding experiments were carried out in the presence of 5 mM Mg^{2+}. (a) Synaptosomal membranes were preincubated in the absence (●) or presence of 5 μM GTP (△) or 5 μM GTPγS (▼) for 30 min at 4°C. The incubation was then started by the addition of [^{35}S]-ATPαS for a further 35 min. The data are expressed as mean obtained from three independent experiments carried out in duplicate; vertical lines show s.e.mean. (b) Scatchard plot of [^{35}S]-ATPαS binding isotherms in the presence of GTP. Values obtained from the binding experiment depicted in (a) were analysed by nonlinear regression by using models with one or two binding sites.

The binding of [^{35}S]-ATPαS and [^{3}H]-α,β-MeATP to synaptosomal membranes from rat brain cortex was compared with the high affinity binding of [^{35}S]-UTPαS at a concentration of 1 nM in equilibrium binding experiments. As can be seen in Figure 4, binding to synaptosomal membranes showed a similar density of specific high affinity binding sites for ATPαS and for the P_{2X}-selective ligand α,β-MeATP, whereas that for UTP was much smaller (1/3 of those for ATPαS or α,β-MeATP).

As signal transduction of metabotropic P_2-purinoceptors is mediated by G-proteins we tested whether the binding of [^{35}S]-ATPαS to synaptosomal membranes was affected by GTP or GTPγS. These experiments are usually carried out in the presence of millimolar Mg^{2+}-concentrations. For this purpose the effect of Mg^{2+} was determined first. The addition of 5 mM Mg^{2+} to the binding buffer significantly increased [^{35}S]-ATPαS binding affinity (Table 3). Analysis of the data for displacement of [^{35}S]-ATPαS binding by unlabelled ATPαS in the presence of 5 mM Mg^{2+} revealed that the affinity of the high affinity binding site was increased about 10 fold (K_d 1.8 nM versus 21 nM in the absence of Mg^{2+}). The affinity of [^{35}S]-ATPαS in synaptosomes preincubated with 5 μM GTP or 5 μM GTPγS (together with 5 mM Mg^{2+}) was strongly decreased (Figure 5 and Table 3). Scatchard plots of the [^{35}S]-ATPαS binding isothermes under these conditions showed that the K_d for the high affinity binding site was increased about 5 fold (K_d 1.8 nM versus 8.6 nM) as compared to the control values in the presence of Mg^{2+} (Figure 5b and Table 3). Hill slopes of the competition curves for ATPαS in the presence ($n_H=0.736\pm0.124$; $n=3$) and absence of GTP/Mg^{2+} ($n_H=0.676\pm0.092$; $n=3$) did not show a significant change.

The guanine nucleotides have a weak direct interaction with the [^{35}S]-ATPαS binding sites, since analysis of the data from the competition experiments revealed K_i values of 7.2 μM and 3.8 μM for GTP and GTPγS, respectively for high affinity [^{35}S]-ATPαS binding (Figure 2 and Table 3). The direct interaction of GTP and GTPγS with the high affinity site was very small since less than 10% reduction of specific [^{35}S]-ATPαS binding was detected in a concentration range up to 100 μM (see Figure

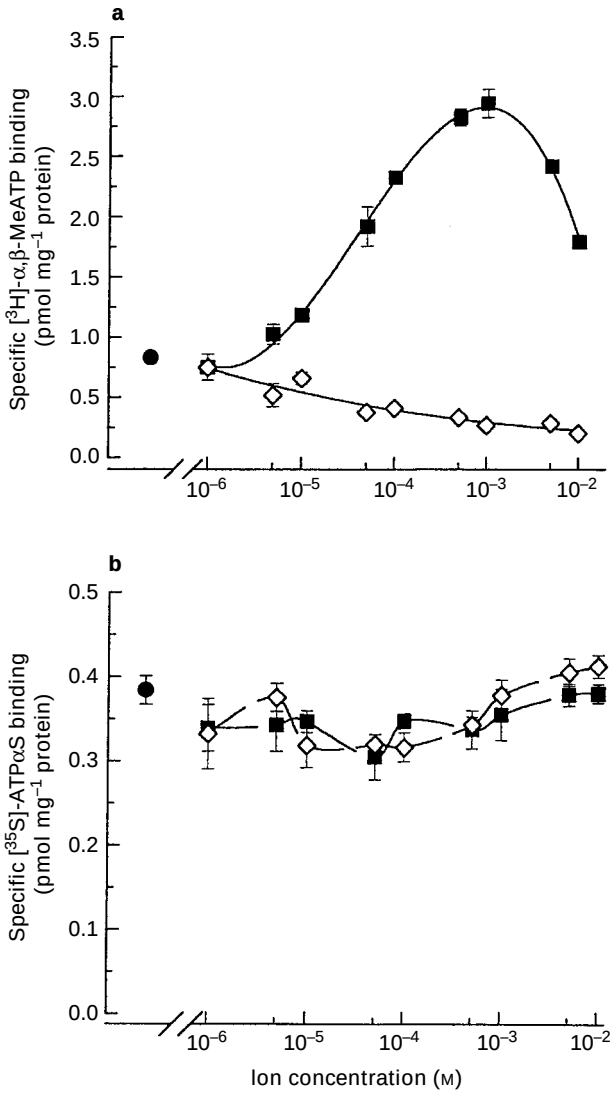

Figure 6 Effect of Ca^{2+} and Mg^{2+} on [^{3}H]-α,β-MeATP binding (a) or [^{35}S]-ATPαS binding (b) to synaptosomes. The binding of 3 nM [^{3}H]-α,β-MeATP or 1 nM [^{35}S]-ATPαS to synaptosomal membranes was determined in the absence (●; control) or the presence of increasing concentrations of Ca^{2+} (■) or Mg^{2+} (◇) for 35 min at 4°C. The data are expressed as mean from three independent experiments performed in duplicate; vertical lines indicate s.e.mean.

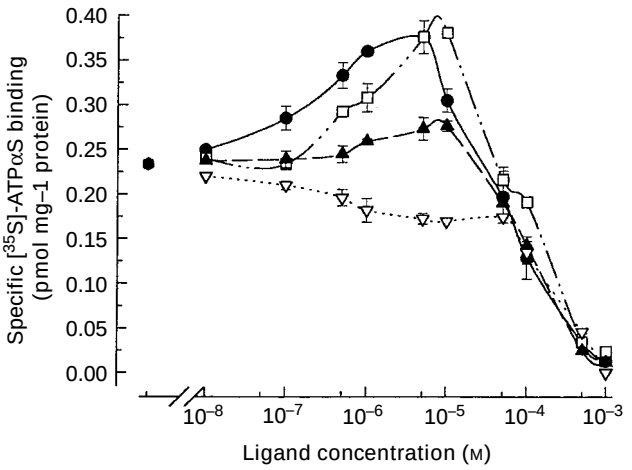

Figure 7 Competition of [^{35}S]-ATPαS binding to synaptosomes by diadenosine polyphosphates. Synaptosomal membranes were incubated with 0.5 nM [^{35}S]-ATPαS in the presence of Ap_3A (▽), Ap_4A (▲), Ap_5A (□) or Ap_6A (●) at the concentrations indicated. The data are expressed as mean from three to four independent experiments performed in duplicate; vertical lines indicate s.e.mean.

2). GTP did not displace [^{3}H]-α,β-MeATP binding in the same range of concentrations (Table 2).

Previous studies demonstrated a marked effect of Ca^{2+} and Mg^{2+} on [^{35}S]-ATPγS or [^{3}H]-α,β-MeATP binding to rat vas deferens membranes (Michel & Humphrey, 1994; 1996). Here the effect of these divalent cations on the binding of the radioligands [^{35}S]-ATPαS or [^{3}H]-α,β-MeATP to synaptosomal membranes was also investigated. The binding of [^{3}H]-α,β-MeATP to synaptosomal membranes increased 3 fold by raising the Ca^{2+} concentration from 10 μM to 1 mM (Figure 6a), while further increasing the Ca^{2+} concentration (from 1 to 10 mM) led to a decline of specific [^{3}H]-α,β-MeATP binding. The addition of Mg^{2+} in the same concentration range resulted in a strong reduction (80%, $n=2$) of the binding (Figure 6a). The reduction by Mg^{2+} of the binding of [^{3}H]-α,β-MeATP could be possibly due to the formation of Mg^{2+}/[^{3}H]-α,β-MeATP complexes, as B_{max} was reduced to 1.28 pmol mg^{-1} protein (as compared to 6.34 pmol mg^{-1} protein) with no change in the affinity ($K_d=14.5$ nM, $n=2$).

In contrast, total [^{35}S]-ATPαS binding was not affected at a range of Ca^{2+} or Mg^{2+} concentrations from 10 μM to 1 mM (Figure 6b), while the addition of Mg^{2+} (5 mM) increased the affinity of [^{35}S]-ATPαS binding to the high affinity site 10 fold (Table 3). Thus, the binding of both radioligands was regulated in an opposite direction by physiological concentrations of Ca^{2+} and Mg^{2+}, respectively.

Diadenosine polyphosphates had been proposed to exert physiological effects through P_2-like purinoceptors in some tissues (Pintor *et al*., 1993; Castro *et al*., 1994; Ralevic *et al*., 1995). We therefore examined whether high affinity [^{35}S]-ATPαS binding was affected by diadenosine polyphosphates (Figure 7). AP_5A and Ap_6A gradually enhanced binding of 1 nM [^{35}S]-ATPαS by up to 60% in a concentration range from 1 to 50 μM. Ap_4A only slightly increased [^{35}S]-ATPαS binding (~15%) over this concentration range. All the diadenosine polyphosphates tested (Ap_3A, Ap_4A, Ap_5A, Ap_6A) inhibited binding of [^{35}S]-ATPαS to synaptosomal membranes (Figure 7), albeit with low potency. For Ap_3A K_i-values of 7.2±1.3 μM ($n=3$) and 240±52 μM ($n=3$) were calculated for the high- and the low-affinity binding site, respectively. The K_i-values for the other diadenosine polyphosphates could not be determined due to the biphasic curves demonstrated in Figure 7.

EctoATPases strongly affect the functional potency of the P_2 purinoceptor agonists and could be a candidate for some of the low affinity binding described here. Therefore we investigated whether specific binding of [^{35}S]-ATPαS or [^{3}H]-α,β-MeATP was affected by the presence of Evan's Blue, a commonly used inhibitor of ectoATPases. Specific binding of 1 nM [^{35}S]-ATPαS was not altered in the presence of Evan's Blue (Figure 8a). There was no shift in the K_i value (19.2 nM, $n=2$) as compared to the competitive potency of ATPαS (21 nM) in the absence of Evan's Blue. In contrast, [^{3}H]-α,β-MeATP binding was reduced by about 40% when membranes were preincubated with 1 μM Evan's Blue (Figure 8b) and the K_i value was shifted slightly from 19±5 nM to 29 nM ($n=2$). These results indicate that neither [^{35}S]-ATPαS nor [^{3}H]-α,β-MeATP binding sites include ectoATPases under our assay conditions and that the low affinity binding sites probably represent some other purine binding proteins.

Discussion

It has become apparent that the various P_2-purinoceptor subtypes play important roles in the regulation of the physiological processes involved in neurotransmission as well as vascular tone, platelet aggregation, secretion or apoptosis (for review see Harden *et al*., 1995). In the CNS the presence of P_{2X}- and P_{2Y}-purinoceptors in synaptosomes has been indica-

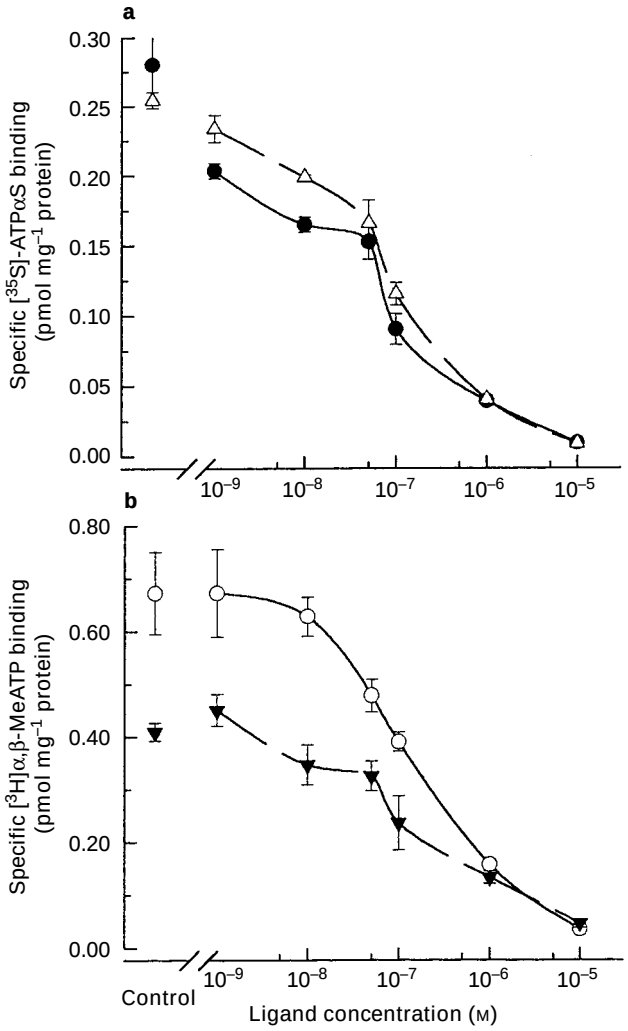

Figure 8 Effect of preincubation of synaptosomal membranes with Evan's Blue on (a) [^{35}S]-ATPαS or (b) [^{3}H]-α,β-MeATP binding. Synaptosomal membranes (25 μg of protein) were used for the binding experiment. (a) Binding of [^{35}S]-ATPαS to membranes with (△) or without (●) pretreatment with Evan's blue (15 min at 4°C). (b) Binding of [^{3}H]-α,β-MeATP to membranes with (▼) or without (○) pretreatment with Evan's blue. The data are expressed as mean values from two experiments performed in triplicate in two different membrane preparations. Vertical lines show s.e.mean.

ted by physiological experiments (Krishtal *et al*., 1988; Evans *et al*., 1992; Edwards *et al*., 1992; Illes & Nörenberg, 1993; von Kügelgen *et al*., 1994; Cloues, 1995; Sun & Stanley, 1996). However, until now no detailed binding characteristics of these P_2 purinoceptors have been published. Synaptosomes appear to be a suitable system amenable to studies investigating binding characteristics and modulation of P_2 purinoceptors.

Here we demonstrate that synaptosomes from rat brain cortex possess different binding sites for [^{35}S]-ATPαS and [^{3}H]-α,β-MeATP. The K_d value of 21 nM for the high affinity [^{35}S]-ATPαS binding site is similar to that obtained in a previous study with a crude membrane fraction from rat brain (Simon *et al*., 1995) where only one binding site with an affinity of 9 nM and a B_{max} of 39 pmol mg^{-1} protein was found, albeit the binding conditions were different. Surprisingly with the [^{35}S]-deoxyATPαS analogue in the crude membrane fraction from whole rat brain no low affinity binding was found, in contrast to the present study. With [^{35}S]-ATPαS we found a low affinity binding site (K_d = 2.6 μM) in synaptosomal membranes.

Based on the present knowledge of the selective binding and/or activation of P_2-purinoceptors by the various compounds we discuss whether the high affinity [^{35}S]-ATPαS binding site has P_{2Y}-purinoceptor-like characteristics. The assumption that low concentrations of [^{35}S]-ATPαS bind to P_{2Y}-purinoceptors is supported by the following findings.

Firstly, the rank order in the displacement potency of high affinity [^{35}S]-ATPαS binding to rat brain synaptosomes by the different agonists and antagonists, known to activate P_2-purinoceptors, show an affinity profile characteristic for P_{2Y}-purinoceptors. The K_i-values of the most potent agonists (ATPαS, ATP, ATPγ, ADPβS) are below or within the range normally found for the EC_{50} values of these compounds for physiological responses. Only 2-MeSATP (K_i = 64 nM) was slightly less potent here than in membrane fractions from rat brain (K_i = 14 nM) or chicken brain (K_i = 34 nM; Simon *et al*., 1995). However, a lower potency in physiological studies or binding experiments of 2-MeSATP, which has long been used as a typical P_{2Y}-purinoceptor agonist, was also obtained. The displacement of [^{35}S]-ADPβS binding by 2-MeSATP was lower than either by ADPβS or β,γ-MeATP in endothelial cells (Wilkinson & Boarder, 1995) and 2-MeSATP was even less potent than α,β-MeATP in rat brain synaptosomes (Pintor *et al*., 1993).

Secondly, the rank order of the different agonists in the pharmacological profile and the high UTP concentration needed to compete for high affinity [^{35}S]-ATPαS binding (K_i = 0.44 μM) makes it very unlikely that the binding sites are P_{2U}- or P_{2T}-purinoceptors (Hourani & Hall, 1994), respectively.

Thirdly, high affinity binding of [^{35}S]-ATPαS to ecto-ATPases, which influence the agonist potencies at the various P_2-purinoceptor subtypes (Picher *et al*., 1996; Ziganshin *et al*., 1996) by hydrolysis of the nucleotides and their analogues, seems most unlikely as demonstrated by the following observations: (i) binding of [^{35}S]-ATPαS was unaltered in synaptosomal membranes preincubated with 1 μM Evan's Blue (see Figure 8a). Evan's Blue had been chosen as it is used to inhibit the degradation of ATP by ectoATPase activity in rat vas deferens at relatively low concentrations and is selective for P_{2X}- versus P_{2Y}-purinoceptors in the guinea-pig taenia coli (Wittenburg *et al*., 1996). Moreover, it has been found to have the highest affinity (IC_{50} = 1.6 μM) among the various P_2-purinoceptor antagonists for [^{35}S]-ATPγS binding sites in rat vas deferens membranes (Michel & Humphrey, 1996); (ii) the experimental binding conditions (incubation at 4°C without the addition of divalent cations and wash of the synaptosomal membrane fraction with EDTA-containing buffer before storage) were designed to exclude metabolism of the nucleotides by ATPases; and (iii) the K_m values (in the presence of Ca^{2+} or Mg^{2+}) of ecto-ATPases for the different purine nucleotides ranged from 10 to 80 μM (Picher *et al*., 1996). In addition, low affinity binding sites for [^{3}H]-α,β-MeATP which had been demonstrated to represent labelling of membrane bound ATPases or ATP-dependent proteins (Michel & Humphrey, 1993; Michel *et al*., 1996) were not detected under our conditions.

Fourthly, high affinity binding of [^{35}S]-ATPαS to synaptosomal membranes was sensitive to guanine nucleotides. The observed decrease in [^{35}S]-ATPαS binding in the presence of 5 μM GTP or GTPγS is very likely due to the interaction of GTP or GTPγS with G proteins coupled to the putative P_{2Y}-purinoceptor but certainly not due to a displacement of the ligand from the high affinity binding site. This conclusion is supported by the following findings: (i) The inhibitory effect of GTP on [^{35}S]-ATPαS binding was only seen in the presence of Mg^{2+} (see for comparison Figures 2 and 5). Mg^{2+} ions decreased the rate of dissociation of GTP from the α-subunits of the G proteins. (ii) Binding of 1 nM [^{35}S]-ATPαS was not fully displaced by GTP even at a concentration of 1 mM and less than 10% reduction of specific [^{35}S]-ATPαS binding was detected at concentrations of 100 μM (Figure 2). Thus, the effect of the guanine nucleotides on [^{35}S]-ATPαS binding was typical for G protein-coupled receptors, where the uncoupling of the G protein by binding of GTP (or GTPγS) decreased agonist affinity. Earlier physiological studies with hepatocytes (Charest *et al*., 1985) or turkey erythrocytes (Boyer *et al*., 1989) showed

guanine nucleotide sensivity in the physiological responses to ATP. This was also seen in binding experiments in turkey erythrocytes (Cooper *et al*., 1989) and in a recent study with rat brain (Simon *et al*., 1995). The 40% reduction of 1 nM [^{35}S]-ATPαS binding by GTPγS/Mg^{2+} (Figure 5) does not mean that 40% of the high affinity ATPαS binding proteins are coupled to G-proteins. It reflects an overall reduction in the binding affinity of [^{35}S]-ATPαS binding sites, probably by way of the GTPγS promoted G-protein dissociation. This partial reduction was certainly not due to the concentration of GTPγS used (5 μM), since this was a more than 1300 fold molar excess as compared to the total [^{35}S]-ATPαS binding sites in the assay. It rather may be due to the fact that only part of the [^{35}S]-ATPαS binding sites were coupled to intact heterotrimeric G-proteins. Very recent studies on the coupling mechanism of G-proteins to the seven transmembrane receptor family support the model that the β,γ-subunits rather than the α-subunits are directly coupled to the receptors (Onrust *et al*., 1997) and by cooperation with the receptor open the nucleotide binding site in the α-subunit.

Fifth, [^{35}S]-ATPαS binding sites were tested to see whether they displayed P_{2X}-purinoceptors. A recent binding study showed that [^{3}H]-α,β-MeATP binding sites of recombinant P_{2X}-purinoceptors expressed in CHO-K1 cells (Michel *et al*., 1996) can be specifically labelled with [^{35}S]-ATPγS or [^{33}P]-ATP. We therefore used [^{3}H]-α,β-MeATP to examine if this ligand labels the same P_{2Y}-like binding sites detected with [^{35}S]-ATPαS in synaptosomal membranes. However, [^{3}H]-α,β-MeATP bound to sites totally different from the P_{2Y}-purinoceptor-like sites detected by [^{35}S]-ATPαS, since the affinity of [^{3}H]-α,β-MeATP was 38 fold lower for these binding sites. Furthermore, the affinity profile of the P_2-purinoceptor agonists at the [^{3}H]-α,β-MeATP binding site clearly distinguished these sites from the sites labelled with [^{35}S]-ATPαS in synaptosomal membranes from rat brain. The non-identity of the binding sites detected with [^{3}H]-α,β-MeATP and [^{35}S]-ATPαS, respectively, becomes further evident by (i) the different binding affinities of the P_2-purinoceptor-selective antagonists, (ii) the opposite regulation of binding by the divalent cations Ca^{2+} and Mg^{2+} in synaptosomal membranes from rat brain cortex, (iii) binding of [^{35}S]-ATPαS in the presence of 0.5 μM α,β-MeATP did not result in a different affinity (K_d = 17.2 nM versus 21.3 nM) or a decreased B_{max} value (B_{max} = 14.2 versus 14.8 pmol mg^{-1} protein), excluding the possibility that higher α,β-MeATP concentrations may displace non-specific [^{35}S]-ATPαS binding, and (iv) the different proportion of [^{3}H]-α,β-MeATP binding sites displaced by ATPαS and [^{35}S]-ATPαS binding displaced by α,β-MeATP, respectively. However, at the moment we cannot exclude binding of [^{35}S]-ATPαS to the α,β-MeATP insensitive P_{2X}-purinoceptors (P_{2X2}, P_{2X4}) form rat brain (Bo *et al*., 1995; Buell *et al*., 1996; Soto *et al*., 1996).

The amounts of high affinity binding of [^{3}H]-α,β-MeATP and [^{35}S]-ATPαS, respectively, which probably represent P_{2X}- and P_{2Y}-purinoceptors in synaptosomal membranes from cortex are comparable, whereas the amount of [^{35}S]-UTPαS binding sites which include putative P_{2U}-purinoceptors is only 1/3. This supports the concept that P_{2Y}- and P_{2X}-purinoceptors play a major role in neural signalling pathways. However, it should be noted that the exact density of each P_2-purinoceptor at synaptic terminals has to be determined under the optimal binding conditions for each receptor.

The synaptosomal [^{3}H]-α,β-MeATP binding sites from rat brain cortex are not only different from the binding sites identified by [^{35}S]-ATPαS but also clearly differ from the high affinity [^{3}H]-α,β-MeATP binding sites in rat vas deferens (Michel & Humphrey, 1994; 1996), a tissue which from functional studies is known to express the P_{2X}-purinoceptor (Burnstock, 1990; Harden *et al*., 1995). There is a different rank order in the competition potency of the P_2-purinoceptor agonists tested and a further difference exists concerning the regulation of [^{3}H]-α,β-MeATP binding by Ca^{2+} and Mg^{2+}. While Ca^{2+} and Mg^{2+} both enhance [^{3}H]-α,β-MeATP binding in rat vas deferens membranes (Michel & Humphrey, 1994), only Ca^{2+} increased binding of [^{3}H]-α,β-MeATP to synaptosomal membranes, whereas the addition of Mg^{2+} resulted in a strong reduction of the binding. The enhancement of [^{3}H]-α,β-MeATP binding by Ca^{2+} in synaptosomal membranes may partly result from promotion of [^{3}H]-α,β-MeATP binding to ectoATPases and other proteins, as indicated by the 4 fold increase in the binding capacity. Nevertheless, the different characteristics of [^{3}H]-α,β-MeATP binding in synaptosomal membranes from cortex and in rat vas deferens possibly reflect the presence of different P_{2X}-purinoceptor subtypes.

With respect to the mechanisms involved in the effects of Ca^{2+} and Mg^{2+} on the binding of [^{3}H]-α,β-MeATP and [^{35}S]-ATPαS, there are principally two possible explanations. It may be either that the complex formed between the divalent cations and the radioligand displays higher affinity (Ca^{2+}/[^{3}H]-α,β-MeATP; Mg^{2+}/[^{35}S]-ATPαS) or lower affinity (Mg^{2+}/[^{3}H]-α,β-MeATP) for the binding proteins than the uncomplexed form and/or that the divalent cations bind directly to the binding proteins, or an associated protein, thereby influencing the affinity of the proteins for the radioligands. The former possibility seems only to be valid for a (Mg^{2+}/[^{3}H]-α,β-MeATP)-complex, which obviously cannot bind to the high affinity binding sites. The assumption that the [^{3}H]-α,β-MeATP binding sites are probably P_{2X}-purinceptors is based on the analogy of the Mg^{2+} effect found in functional studies on P_{2X}-purinoceptors in guinea-pig vas deferens (Fedan *et al*., 1990), where the uncomplexed form of ATP seems to be the preferred agonist.

The divalent cations Ca^{2+} and Mg^{2+} could affect P_2-purinoceptors by binding to the receptor protein, leading to altered physiological responses (Krishtal *et al*., 1988; Bean, 1990; Cloues, 1995). An increased response of a ligand-gated ion channel to ATP in the presence of a divalent cation, Zn^{2+}, has been attributed to the direct action of Zn^{2+} on the P_{2X}-purinoceptor (Cloues, 1995). In addition, there are several studies on the action of Ca^{2+} on ATP-gated ion channels. In rat cultured sensory neurones Ca^{2+} has been demonstrated to inhibit ion channel flux through an ATP-gated ion channel (Krishtal *et al*., 1988), while in dorsal root ganglion neurones the deactivation of an ATP-gated ion channel was drastically slowed down by removal of Ca^{2+} (Bean, 1990). A comparison of the activating effects of Ca^{2+} and Mg^{2+} reveals a cation selectivity where Mg^{2+} selectively activates the putative P_{2Y}-purinoceptor and Ca^{2+} activates the putative P_{2X}-purinoceptor in synaptosomal membranes from rat brain cortex.

The biological effects of diadenosine polyphosphates observed in various cells and tissues have been attributed to interactions either with P_{2Y}-purinoceptors (Castro *et al*., 1994; Schlüter *et al*., 1994; Ralevic *et al*., 1995) or with a separate receptor which is specific for diadenosine polyphosphates (Pintor & Miras-Portugal, 1995). In the nervous tissue they have been related to P_{2Y}-purinoceptors (Stone & Perkins, 1981; Krishtal *et al*., 1988). In synaptosomes from rat brain cortex all diadenosine polyphosphates tested showed a low affinity for the P_{2Y}-purinoceptors which are included in the high affinity [^{35}S]-ATPαS binding sites (Figure 7). Our results are consistent with the assumption that synaptosomes possess separate receptors specific for diadenosine polyphosphates, as deduced recently from Ca^{2+} flux experiments in a synaptosomal preparation from rat midbrain (Pintor & Miras-Portugal, 1995). High affinity binding sites specific for Ap_4A on rat brain synaptosomes were demonstrated by Pintor *et al*. (1993). The reason for the enhancement of [^{35}S]-ATPαS binding to synaptosomal membranes by Ap_5A and Ap_6A in the concentration range of 1 to 100 μM is not yet known, but could possibly be due to a cooperative effect on [^{35}S]-ATPαS binding to the high affinity binding site. The effects of the diadenosine polyphosphates on [^{35}S]-ATPαS binding do not result from inhibition of ecto-ATPase activity since (i) the characteristics of high affinity ATPαS binding sites rule out binding to ecto-ATPases (see above), and (ii) no detectable metabolism of [^{35}S]-ATPαS under our binding assay conditions could be ob-

served (<15% of [^{35}S]-ATPαS was degraded after incubation for 45 min at 0°C; data not shown).

In summary, specific high affinity [^{35}S]-ATPαS binding sites occur at a high density on synaptosomes from rat brain cortex. The results strongly indicate that the high affinity [^{35}S]-ATPαS binding sites are to a large extent P_{2Y}-purinoceptors, as seen by the high affinity of ATP or its derivatives, the lack of competition by UTP, binding characteristics totally different from those of [^{3}H]-α,β-MeATP binding sites and the GTP sensitivity. Thus, [^{35}S]-ATPαS is suitable to study further in detail P_{2Y}-purinoceptors in rat brain. The specific binding of [^{35}S]-ATPαS and [^{3}H]-α,β-MeATP to synaptosomal membranes was differently regulated by Ca^{2+} and Mg^{2+}. Therefore, neural signalling by P_{2Y}- or P_{2X}-purinoceptors in brain synaptosomes is possibly modulated by divalent cations.

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