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Ap_4A and ADP-β-S binding to P_2 purinoceptors present on rat brain synaptic terminals

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1 Diadenosine tetraphosphate (Ap_4A) a dinucleotide stored and released from rat brain synaptic terminals presents two types of affinity binding sites in synaptosomes. When [^{3}H]-Ap_4A was used for binding studies a K_d value of 0.10 ± 0.014 nM and a B_{max} value of 16.6 ± 1.2 fmol mg^{-1} protein were obtained for the high affinity binding site from the Scatchard analysis. The second binding site, obtained by displacement studies, showed a K_i value of 0.57 ± 0.09 μM.

2 Displacement of [^{3}H]-Ap_4A by non-labelled Ap_4A and P_2-purinoceptor ligands showed a displacement order of Ap_4A > adenosine 5'-O-(2-thiodiphosphate) (ADP-β-S) > 5'-adenylyl-imidodiphosphate (AMP-PNP) > α,β-methylene adenosine 5'-triphosphate (α,β-MeATP) in both sites revealed by the K_i values of 0.017 nM, 0.030 nM, 0.058 nM and 0.147 nM respectively for the high affinity binding site and values of 0.57 μM, 0.87 μM, 2.20 μM and 4.28 μM respectively for the second binding site.

3 Studies of the P_2-purinoceptors present in synaptosomes were also performed with [^{35}S]-ADP-β-S. This radioligand showed two binding sites the first with K_d and B_{max} values of 0.11 ± 0.022 nM and 3.9 ± 2.1 fmol mg^{-1} of protein respectively for the high affinity binding site obtained from the Scatchard plot. The second binding site showed a K_i of 0.018 ± 0.0035 μM obtained from displacement curves.

4 Competition studies with diadenosine polyphosphates of [^{35}S]-ADP-β-S binding showed a displacement order of $Ap_4A > Ap_5A > Ap_6A$ in the high affinity binding site and K_i values of 0.023 nM, 0.081 nM and 5.72 nM respectively. The second binding site potency order was $Ap_5A > Ap_4A > Ap_6A$, with K_i values of 0.28 μM, 0.53 μM and 5.32 μM respectively.

5 Displacement studies of [^{35}S]-ADP-β-S with P_2-purinoceptor agonists showed the following potency pattern: ADP-β-S > AMP-PNP > α,β-MeATP with K_i values of 0.021 nM, 0.029 nM 0.215 nM respectively in the high affinity binding site. 2-Methylthio-adenosine 5'-triphosphate (2MeSATP) was unable to displace [^{35}S]-ADP-β-S in this binding site. The second binding site showed a profile of ADP-β-S > α,β-MeATP > AMP-PNP > 2MeSATP and K_i values of 0.018 μM, 0.212 μM, 0.481 μM and 18.04 μM respectively.

6 These studies suggest the presence of a new P_2-purinoceptor in rat brain synaptosomes with high affinity for diadenosine polyphosphates which we tentatively designate as P_{2d}.

Keywords: Adenosine-(5')-tetraphospho-(5')-adenosine (Ap_4A); diadenosine polyphosphates; P_{2d}-purinoceptors; synaptosome

Introduction

Since the idea that purinergic nerves exist was first conceived, intensive studies of the actions of adenosine and ATP, through plasma membrane receptors, have been carried out (Burnstock, 1972; Gordon, 1986; Williams, 1987). Separate receptors for adenosine and ATP were soon recognized and classified as P_1- and P_2-purinoceptors respectively (Burnstock, 1978). Subsequently, four different classes of P_2-purinoceptors have been proposed. P_{2x} and P_{2y} were the first to be found, based on the divergent effects of ATP and analogues, on vascular and visceral smooth muscles (Burnstock & Kennedy, 1985), P_{2t}-purinoceptors, the main natural agonist of which is ADP, are involved in platelet aggregation. P_{2z}-purinoceptors are linked to mast cell stimulation and ATP^{4-} is the active ligand (Cockcroft & Gomperts, 1980).

In spite of the broad studies in the field of P_2-purinoceptors, no potent, selective and reversible antagonists have yet been identified, but even in their absence the existence of other P_2-purinoceptors seems probable. For example, recently a receptor with different agonist order from that already described, has been reported (O'Connor *et al.*, 1991).

The presence of diadenosine polyphosphates as natural constituents of secretory granules, increases the diversity of secreted purinergic compounds and their possibilities of action as extracellular signals. The discovery of Ap_4A and Ap_5A, and more recently Ap_6A, in chromaffin cells increases the possibility of their role as neural transmitters and modulators (Rodriguez del Castillo *et al.*, 1988; Pintor *et al.*, 1992c). In this neural model, the release of these dinucleotides after carbachol stimulation allowed their quantification in the extracellular media at concentrations in the μM range (Pintor *et al.*, 1991a). It has also been possible to characterize receptors for Ap_xA with the highest affinities described for a nucleotide ($K_d \approx 10^{-11}$ M) (Pintor *et al.*, 1991b). Ap_4A and Ap_5A acting through P_{2y}-receptors are able to inhibit the catecholamine secretion induced by nicotine (Castro *et al.*, 1990). These purinoceptors subserve increases in the intracellular levels of Ca^{2+} when they are stimulated by diadenosine polyphosphates (Castro *et al.*, 1992).

This is not the only model in which diadenosine polyphosphates show biological actions. Ap_3A and Ap_4A, stored in dense secretory granules, inhibit platelet aggregation (Cusack & Hourani, 1982; Flodgaard & Klenow, 1982; Lüthje & Ogilvie, 1983; Zamecnick *et al.*, 1992). Macrophage membranes, which possess P_{2z}-purinoceptors, are susceptible to diadenosine pentaphosphate, inducing the typical pore formation on these membranes (Steinberg *et al.*, 1987). In other organs, such as the liver, diadenosine polyphosphates are able to displace ATP-γS from P_2-purinoceptors present on liver membranes (Keppens *et al.*, 1989). Noteworthy are the actions of Ap_4A and Ap_5A on vas deferens, which has similar effects to the potent P_{2x} agonist, α,β-MeATP (MacKenzie *et al.*, 1988). Ap_6A has also been found to act on the urinary bladder via P_{2x}-purinoceptors (Hoyle *et al.*, 1989). Studies

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have been carried out in sensory neurones where the effects of different nucleotides and diadenosine polyphosphates, have been reported. These compounds activate a receptor which opens cation channels with low ionic selectivity and strong inward-going rectification (Krishtal *et al.*, 1988).

The presence of diadenosine polyphosphates in the synaptosomes of rat brain, and their release by depolarizing agents, emphasized the importance of these substances as possible neurotransmitters in the central nervous system and invites the search for their physiological actions mediated by membrane receptors (Pintor *et al.*, 1992a).

The present paper deals with the existence of specific receptors for Ap_xA in brain synaptic terminals, and also the classification of these purinoceptors by use of different ATP analogues, in order to establish similarity to or difference from the P_2-purinoceptors previously described.

Methods

Synaptosomal preparation

Synaptosomes were prepared from middle cerebral cortices of male Wistar rats cervically dislocated and decapitated as described by Pintor *et al.* (1992a). Synaptosomes in 1 or 10 mg pellets, were resuspended into 1 ml incubation medium (composition mM: NaCl 122, KCl 3.1, KH_2PO_4 0.4, $NaHCO_3$ 5, $MgSO_4$ 1.2, glucose 10 and TES buffer 20, pH 7.4).

High performance liquid chromatography (h.p.l.c.) procedures

The h.p.l.c. equipment consisted of a Spectra Physics SP 8800 ternary pump, a Spectra 100 variable wavelength detector, a Reodyne injector and a Chrom Jet integrator. The column was an RP-18 (22 cm length, 0.46 mm diameter) from Brownlee (Applied Biosystems). The conditions of the mobile phase were as follows: KH_2PO_4 10 mM, tetrabutylammonium 2 mM, 10% acetonitrile, pH 7.5.

Concentrations of 1 μM diadenosine polyphosphates and ATP analogues were assayed in 1 ml of incubation media with 1 mg of synaptosomes ml^{-1}. Samples of 25 μl were taken for h.p.l.c. analysis.

Binding studies with [^{3}H]-Ap_4A

[^{3}H]-Ap_4A (6.3 Ci $mmol^{-1}$) binding assays to rat brain synaptosomes were performed with 0.6 mg of protein synaptosomal preparations in 1 ml of the incubation medium (described under 'synaptosomal preparation') and graded concentrations of [^{3}H]-Ap_4A, ranging from 0.025 nM to 2.5 nM. In order to obtain the saturation isotherms, the incubation conditions were 45 min at 25°C temperature. Binding was stopped by filtering through Whatman GF/C filters and washing with 10 ml of cold incubation medium containing 10 μM cold Ap_4A. The filters were then put into scintillation vials, with 5 ml of Beckman Ready Safe scintillation cocktail. Finally the vials were counted in a Beckman LS 3801.

In order to quantify the non-specific binding, the same procedure was carried out in the presence of 10 μM cold Ap_4A.

Displacement studies were performed with 0.6 mg of synaptosomes in a final volume of 1 ml incubation medium and in the presence of a concentration of 0.25 nM [^{3}H]-Ap_4A. P_2-purinoceptor agonists as well as Ap_4A were assayed in concentrations ranging from 10^{-12} to 10^{-3} M.

Binding studies with [^{35}S]-ADP-β-S

[^{35}S]-ADP-β-S (1242 Ci $mmol^{-1}$) binding assays to synaptic terminals were studied with 50 μg of synaptosomes in 1 ml of the incubation medium, as previously described, and in the presence of graded concentrations of [^{35}S]-ADP-β-S ranging from 0.025 nM to 2.5 nM. The incubation conditions were 45 min at 25°C. When necessary the binding experiments were performed at 4°C with 4 h incubation time. Binding was stopped by addition of 10 ml of cold incubation medium with 10 μM cold ADP-β-S after filtering through Whatman GF/C filters following the same protocol as described for [^{3}H]-Ap_4A.

Non-specific binding was evaluated by incubation in the presence of 10 μM ADP-β-S.

Competition binding assays were initiated with 50 μg of synaptosomes in 1 ml of incubation medium containing [^{35}S]-ADP-β-S 0.1 nM. Diadenosine polyphosphates and P_2-purinoceptor agonists were assayed in a concentration ranging from 10^{-12} to 10^{-3} M. 2MeSATP was assayed at 4°C for 4 h to avoid possible ectonucleotidease activities.

Computer analysis

Data from saturation and displacement experiments were processed, covering a range from 10^{-12} to 10^{-3} M Ap_4A and ADP-β-S. Binding curves were fitting directly to raw c.p.m. data, to avoid biasing during intermediate calculations, with the aid of the programmes EQUIL, kindly provided by Dr. R.F. Goldstein, Computer Center, Univ. Illinois of Chicago (Goldstein & Leung, 1990) and LIGAND (Munson & Rodbard, 1980). The equilibrium parameters were determined by the equations described by Casadó *et al.* (1990) to avoid the interference of non-specific binding by the inclusion of the corresponding unknown parameter in the equations. All the results obtained are means ± s.d.

Materials

[^{3}H]-Ap_4A (6.3 Ci $mmol^{-1}$) was purchased from Amersham (United Kingdom). [^{35}S]-ADP-β-S (1242 Ci $mmol^{-1}$) was from New England Nuclear (United Kingdom). All nucleotides and dinucleotides were obtained from Boehringer (Manheim, Germany) except Ap_6A which was purchased from Sigma (St. Louis, U.S.A.) and 2MeSATP which was purchased from RBI (U.S.A.). Other reagents were analytical grade purchased from Merck (Darmstadt, Germany).

Results

H.p.l.c. analysis

ATP analogues as well as diadenosine polyphosphates were analysed by h.p.l.c. as described in Methods, in order to verify their structure during the incubation period. Neither diadenosine polyphosphates nor ATP analogues were affected during the incubation period. As shown in Figure 1, Ap_4A and ADP-β-S were stable during the whole incubation time; the same procedure was followed with the other nucleotides and dinucleotides. 2MeSATP was incubated for 4 h at 4°C to avoid possible ectonucleotidase activity. This time is sufficient to reach the equilibrium for binding studies. H.p.l.c. analysis confirmed its stability during the incubation period (not shown). These results allowed us to develop the following binding experiments with the assurance of the stability of all nucleotides.

[^{3}H]-Ap_4A binding to rat brain synaptic terminals

[^{3}H]-Ap_4A data from six experiments were transformed and represented as a Scatchard plot (Figure 2). The results were analysed by the method for binding parameters estimation of Casadó *et al.* (1990). The results of computer studies showed the presence of a high affinity binding site in the nM range, with a K_d value of 0.10 ± 0.014 nM and a B_{max} value of 16.6 ± 1.2 fmol mg^{-1} of protein. The curvilinear profile in the

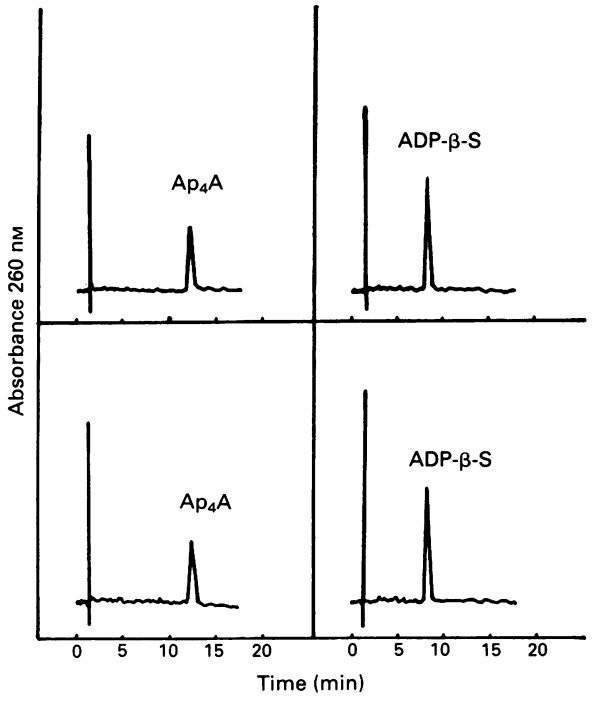

Figure 1 High performance liquid chromatography (h.p.l.c.) of Ap_4A and ADP-β-S stability during the binding study: (a) h.p.l.c. elution profile of 25 pmol Ap_4A after incubation with 1 mg synaptosomes ml^{-1} for 45 min under the conditions described in Methods; (b) h.p.l.c. elution profile of 25 pmol of standard Ap_4A; (c) elution profile of 25 pmol ADP-β-S after incubation for 45 min with 1 mg synaptosomes ml^{-1} under the conditions described in Methods; (d) elution profile of 25 pmol standard ADP-β-S. For abbreviations in this and subsequent legends, see text.

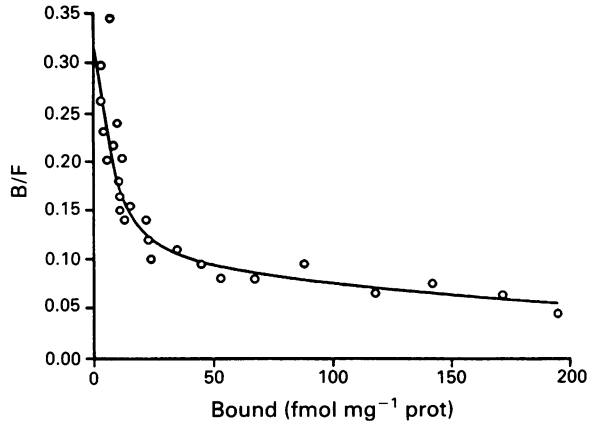

Figure 2 Scatchard analysis of equilibrium of [^{3}H]-Ap_4A binding to rat brain synaptosomes. Binding was studied with 0.6 mg synaptosomes ml^{-1} as described in Methods. K_d and B_{max} values were obtained by the method of Casadó *et al.* (1990). Results are means of six experiments in duplicate.

experimental concentration-range employed, is a consequence of an increasing non-specific binding, which is a difficult experimental value to obtain and in this way, it was included as an unknown parameter in the K_d determination equations (Casadó *et al.*, 1990).

[^{3}H]-Ap_4A competition studies with Ap_4A and P_2 agonists

Indirect binding assays were carried out with non-labelled Ap_4A and different P_2 agonists in order to characterize the pharmacological profile of the sites present in the synaptosomes.

In Figure 3 the displacement curves for Ap_4A, ADP-β-S, α,β-MeSATP and AMP-PNP are shown (results of four experiments in duplicate). The percentage displacement in the first binding site was approximately 15% of the total for the four compounds assayed. Data from saturation and displacement studies were processed together and fitted with the help of the computer programmes EQUIL and LIGAND (Goldstein & Leung, 1990; Munson & Rodbard, 1980) to obtain the K_i values. The displacement binding parameters (K_i) for the first and second steps are summarized in Table 1. The displacement of [^{3}H]-Ap_4A by non-labelled Ap_4A shows two different binding sites. The first was in the nM range with a K_i in the order of the K_d obtained in the Scatchard plot. The second was in the μM range and it was not detectable by Scatchard analysis in the experimental radioligand concentration.

The displacement order for the first binding site was $Ap_4A > ADP\text{-}\beta\text{-}S > AMP\text{-}PNP > \alpha,\beta\text{-}MeATP$. The second binding site showed a potency order of $Ap_4A > ADP\text{-}\beta\text{-}S > AMP\text{-}PNP > \alpha,\beta\text{-}MeATP$. The Hill numbers ($n_H$) for these compounds (less than 1) suggested the presence of more than one binding site.

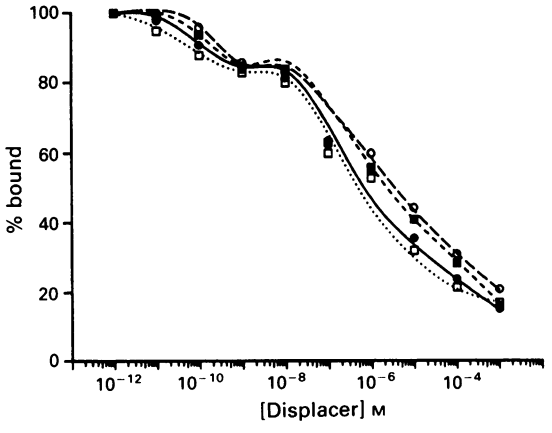

Figure 3 Displacement studies of radiolabelled diadenosine tetraphosphate ([^{3}H]-Ap_4A) by cold Ap_4A and P_2-receptor agonists. Synaptosomes in a concentration of 0.6 mg ml^{-1} were incubated in the presence of Ap_4A (□), ADP-β-S (●), AMP-PNP (■) and α,β-MeATP (○). All the experiments represented the mean of four experiments in duplicate. The K_i values are summarized in Table 1.

Table 1 K_i values for the [^{3}H]-diadenosine tetraphosphate ([^{3}H]-Ap_4A) binding displacement by diadenosine tetraphosphate and P_2 agonists

	[^{3}H]-Ap_4A		
Compound	K_{i_1} (nM)	K_{i_2} (μM)	n_H
Ap_4A	0.017 ± 0.004	0.57 ± 0.09	0.26
ADP-β-S	0.030 ± 0.006	0.87 ± 0.12	0.23
AMP-PNP	0.058 ± 0.006	2.20 ± 0.83	0.25
α,β-MeATP	0.147 ± 0.031	4.28 ± 1.03	0.24

Binding parameters for [^{3}H]-Ap_4A: K_d: 0.010 ± 0.014 nM; B_{max}: 16.6 ± 1.2 fmol mg^{-1} protein.
K_i values for Ap_4A and P_2 purinoceptor agonists correspond to Figure 2. The K_i values were calculated by the programme EQUIL (Goldstein & Leung, 1990) which adjusted data to non-linear weighted regressions. K_{i_1} and K_{i_2} correspond to the very high affinity and the high affinity binding sites respectively.
For abbreviations, see text.

Characaterization of the synaptic terminal binding sites with [35S]-ADP-β-S

[35S]-ADP-β-S, one of the most potent P_{2Y}-agonists, helped us to identify the binding sites present in synaptosomes. When the saturation studies were performed ($n = 6$), the Scatchard plot profile observed was very similar to that obtained with [3H]-Ap_4A (Figure 4). In the concentration-range used to obtain the equilibrium isotherms a curvilinear plot was obtained as a consequence of an increasing of non-specific binding. To avoid this interference, data were fitted to the equations described by Casadó *et al.* (1990). The results of these studies showed the presence of one binding site with a K_d value of 0.11 ± 0.022 nM, and a B_{max} of 3.9 ± 2.1 fmol mg^{-1} of protein.

[35S]-ADP-β-S displacement studies with diadenosine polyphosphates

The ability of Ap_4A, Ap_5A and Ap_6A to displace [35S]-ADP-β-S, was assayed with a concentration value in the order of its K_d value. In Figure 5 the behaviour of these compounds in displacing [35S]-ADP-β-S is shown (results of 4 experiments in duplicate). The K_i values for these dinucleotides in both binding sites, calculated with the help of the computer programmes, are summarized in Table 2. Ap_4A was the best competitor at the high affinity binding site but not in the second. On the other hand, Ap_5A was more active in displacing at the second site than any other dinucleotide. Ap_6A was the less potent compound at both sites.

[35S]-ADP-β-S competition studies with P_2 agonists

Looking for a pharmacological profile of the binding sites present in rat brain synaptic terminals, we performed competition studies of [35S]-ADP-β-S with ADP-β-S, α,β-MeATP, AMP-PNP and 2MeSATP (results of 4 experiments in duplicate). As shown in Figure 6, the P_2 pattern displacement was ADP-β-S > AMP-PNP > α,β-MeATP. It is necessary to emphasize that 2MeSATP was not able to displace [35S]-ADP-β-S from the high affinity binding site. The displacement order for the second binding site was: ADP-β-S > α,β-MeATP > AMP-PNP > 2MeSATP. This agonists order was different from any known classification for P_2 purinoceptors.

The K_i values for the four compounds and for both sites are summarized in Table 2. All the compounds appear to bind to more than one site, except for 2MeSATP with a Hill number close to 1.

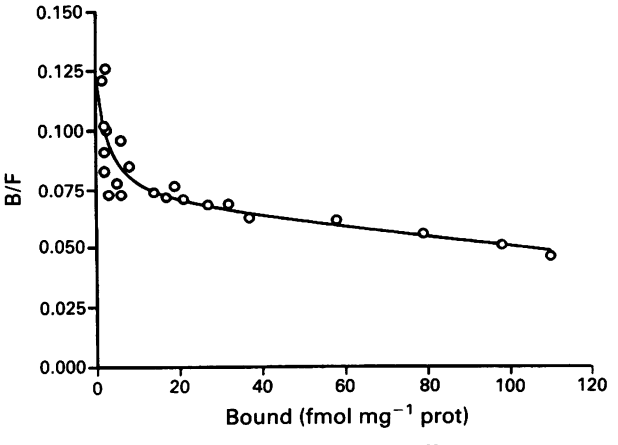

Figure 4 Scatchard analysis of equilibrium of [35S]-ADP-β-S binding to rat brain synaptosomes. Binding was studied with 50 μg synaptosomes ml^{-1} as described in Methods. K_d and B_{max} values were obtained by the method of Casadó *et al.* (1990). Results are means of six experiments in duplicate.

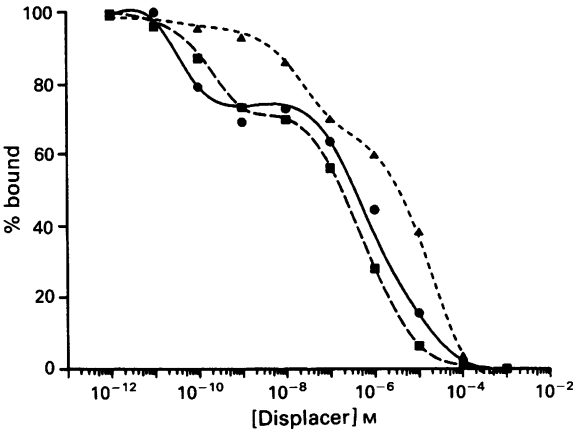

Figure 5 Inhibition of site specific binding of [35S]-ADP-β-S to rat brain synaptosomes. Synaptosomes were incubated in a concentration of 50 μg ml^{-1} in the presence of [35S]-ADP-β-S as described in Methods in the presence of each Ap_4A (●), Ap_5A (■) and Ap_6A (▲). Results are plotted as a percentage of site specific binding of [35S]-ADP-β-S in the absence of inhibitors. Results are means of four experiments in duplicate.

Table 2 K_i values for the [35S]-adenosine 5'-O-(2 thiodiphosphate ([35S]-ADP-β-S) binding displacement by diadenosine polyphosphates and P_2 receptor agonists

	[35S]-ADP-β-S		
Compound	K_{i_1} (nM)	K_{i_2} (μM)	n_H
Ap_4A	0.023 ± 0.007	0.53 ± 0.012	0.30
Ap_5A	0.081 ± 0.012	0.28 ± 0.027	0.36
Ap_6A	5.72 ± 0.85	5.32 ± 0.97	0.41
ADP-β-S	0.021 ± 0.007	0.018 ± 0.003	0.33
AMP-PNP	0.029 ± 0.008	0.481 ± 0.092	0.43
α,β-MeATP	0.215 ± 0.035	0.212 ± 0.046	0.62
2MeSATP	–	18.04 ± 3.72	0.86

Binding parameters for [35S]-ADP-β-S: K_d: 0.11 ± 0.022 nM; B_{max}: 3.9 ± 2.1 fmol mg^{-1} protein.
K_i values for diadenosine polyphosphates and P_2 purinoceptor agonists correspond to Figures 5 and 6. The K_i values were calculated by the computer programme EQUIL (Goldstein & Leung, 1990) which adjusted data to non-linear wieghted regressions. K_{i_1} and K_{i_2} correspond to the very high affinity and the high affinity binding sites respectively.
For abbreviations, see text.

As described in Methods, 2MeSATP experiments were performed at 4°C. To verify that temperature did not alter the binding features, i.e. as a consequence of lipid-phase transitions, binding experiments with [35S]-ADP-β-S were also performed at 4°C. Under these conditions the K_d and B_{max} values were respectively 0.083 ± 0.0015 nM and 3.0 ± 1.2 fmol mg^{-1} protein, being the ratio K_d/B_{max} 27600. The quotient K_d/B_{max} at 25°C was 28205 (see Table 2). These data suggest that the behaviour of 2MeSATP is not due to a problem with the temperature.

Discussion

In the present experimental work we show that diadenosine polyphosphates, which are stored and released from rat brain synaptosomes, can bind to P_2 purinoceptors present in these synaptic terminals. Two radioligands, [3H]-Ap_4A and [35S]-ADP-β-S were used to perform the binding studies.

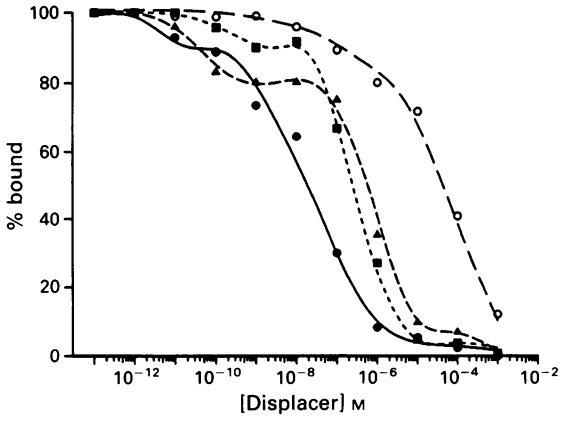

Figure 6 Competition studies of [^{35}S]-ADP-β-S binding by P_2 purinoceptor agonists. Synaptosomes were incubated in the presence of [^{35}S]-ADP-β-S as described in Methods. Synaptosomes in a concentration of 50 μg ml^{-1} were incubated in the presence of ADP-β-S (●), AMP-PNP (▲), α,β-MeATP (■) and 2MeSATP (○). All the experiments represent the mean of four experiments in duplicate.

The binding experiments carried out with [^{3}H]-Ap_4A showed the presence of two binding sites in middle brain synaptic terminals after data analysis. In chromaffin cells where [^{3}H]-Ap_4A binding studies have been done, two binding sites have also been characterized (Pintor *et al.*, 1991b). The high affinity binding site dissociation constant in synaptic terminals (K_d = 0.10 nM) was in the same order as that found in chromaffin cells (K_d = 0.080 nM). The second binding site, obtained from displacement studies, in the synaptosomes had a K_i value close to that described with respect to that of chromaffin cells (K_i = 0.13 μM).

Displacement studies of [^{3}H]-Ap_4A by Ap_4A as well as the ATP analogues showed, at the high affinity binding site, a similar pharmacological profile in rat brain synaptic terminals to that in chromaffin cells: Ap_4A > ADP-β-S > AMP-PNP > α,βMeATP (Figure 3). Furthermore, the inhibition constants obtained from the displacement curves were similar for each compound in both systems. The second binding site also showed a similar displacement profile to that described in chromaffin cells: Ap_4A > ADP-β-S > AMP-PNP > α,β-MeATP. Nevertheless, all the K_i values in middle brain synaptosomes were approximately five times higher than those described in cultured chromaffin cells.

[^{35}S]-ADP-β-S is a good radioligand to characterize P_{2y}-purinoceptors (Cooper *et al.*, 1989). Since diadenosine polyphosphates bind to a putative P_{2y}-purinoceptor in chromaffin cells, it was decided to study further the purinoceptors present in rat brain synaptosomes with this labelled ligand.

Binding studies performed with [^{35}S]-ADP-β-S also showed two binding sites on rat brain synaptosomes. The K_d value for the high affinity binding site was similar to that obtained with [^{3}H]-Ap_4A, both in the nanomolar range. The second binding site, calculated from the displacement studies, showed a K_i value in the same range as described in the literature (Williams, 1987; Cooper *et al.*, 1989; Keppens *et al.*, 1989).

The competition studies carried out with diadenosine polyphosphates, using [^{35}S]-ADP-β-S as radioligand, showed an affinity profile Ap_4A > Ap_5A > Ap_6A for the high affinity binding site. In chromaffin cells at the high affinity binding site the three polyphosphates displace the [^{3}H]-Ap_4A binding with the same potency (Pintor *et al.*, 1991b). In this case there is a clear difference in the displacement potency order which is not surprising with different neural models and different animal species. It is generally assumed that a typical P_{2y} profile shows the following order: 2MeSATP > ADP-β-S > AMP-PNP > α,β-MeATP, and a P_{2x}-purinoceptor profile an order of α,β-MeATP > AMP-PNP > ADP-β-S > 2MeSATP (Burnstock, 1991). When the pharmacological analysis using synthetic analogues is performed, synaptosomal binding sites do not seem to belong either to the P_{2y}, or to the P_{2x}. As 2MeSATP is not the most potent displacer, it is difficult to classify the high affinity binding site as a P_{2y}, in spite of the order of the other ligands (Figure 6). The results obtained with ATP synthetic analogues for the second binding site was different from any P_2 classification. At this site the most potent synthetic displacer was ADP-β-S followed by α,β-MeATP and AMP-PNP. The least potent agonist was 2MeSATP which was two orders of magnitude lower in potency than the other ligands (Figure 6).

The relevance of the P_2-purinoceptors with respect to the high affinity for diadenosine polyphosphates is due to the presence of these compounds and their calcium-dependent release from rat brain synaptic terminals (Pintor *et al.*, 1992a). These dinucleotides have recently been described in catecholaminergic neurones (Pintor *et al.*, 1992c) and in pure cholinergic vesicles from *Torpedo* electric organ (Pintor *et al.*, 1992b). Thus, the presence of P_2-purinoceptors, with high affinity for diadenosine polyphosphates, suggests the possibility of important physiological roles of these compounds in the central nervous system, as reported by Stone & Perkins (1981). They demonstrated that ATP, Ap_3A, and Ap_5A were able to depress the firing of spontaneously active cortical neurones. In cortex slices preloaded with [^{3}H]-adenosine, dinucleoside polyphosphates enhanced the spontaneous efflux of the labelled nucleoside. It is possible that those actions are mediated by the P_2-purinoceptors described in the present study. To support this possibility Tschöpl *et al.* (1992), working with *locus ceruleus* neurones, have demonstrated the presence of a P_2-purinoceptor with an agonist order α,β-MeATP > 2MeSATP, which is in accordance with our second binding site. Although these authors tried to classify the purinoceptor as a P_{2x}, the typical desensitization carried out by α,β-MeATP did not occur. The EC_{50} values obtained in these neurones, in the micromolar range, are similar to the K_i values described in the present work (Table 2).

Actions of ATP and diadenosine polyphosphates have been described in the peripheral nervous system and its innervated tissues. Rat nodose ganglia possess purinoceptors which bind ATP and Ap_xA (Krishtal *et al.*, 1988). These binding sites are coupled to a cation channel which is open to allow low specific inward currents.

In vas deferens and urinary bladder, diadenosine polyphosphates acting through P_{2x}-purinoceptors present the same features as α,β-MeATP (Hoyle, 1990).

Synaptosomes join a heterogeneous population of synaptic terminals. They can exhibit different ecto-enzymes and can store different neurotransmitters in their synaptic vesicles (Richardson *et al.*, 1987). In a pure catecholaminergic model, the chromaffin cell, P_2-purinoceptors with high affinity for diadenosine polyphosphates have been described (Pintor *et al.*, 1991b). In the cholinergic model of *Torpedo* synaptosomes the presence of P_2-purinoceptors has been demonstrated which also bind Ap_xA compounds with K_d values very similar to those described in the present work (Pintor & Miras-Portugal, 1993). The possible existence of these purinoceptors in other neurones, such as GABAergic or glutamatergic, ought to be taken into consideration. With our experimental model, it is not possible to know whether P_2-purinoceptors are present or not in all synaptic terminals. Moreover, Inoue *et al.* (1992), have described the modulation of glutamate release by ATP in cultured hippocampal neurones. These findings support the hypothesis of a wide P_2-purinoceptor distribution.

Our findings demonstrate that diadenosine polyphosphates can bind to synaptosomal P_2 purinoceptors. The pharmacological profiles with respect to the Ap_4A and ADP-β-S displacement studies do not agree with any specific P_2-purinoceptor subtype. These observations may relate to those made by Hoyle (1990), who demonstrated different phar-

macological properties of diadenosine polyphosphates in purinergically innervated tissues, suggesting the possible existence of a purinoceptor highly specific for adenine dinucleotides for which we suggest the designation, P_{2d}.

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