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Comparative studies on the affinities of ATP derivatives for P_{2X}-purinoceptors in rat urinary bladder

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1 Radioligand binding assays have been used to determine the affinities of a series of ATP derivatives with modifications of the polyphosphate chain, adenine and ribose moieties of the ATP molecule for [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) binding sites in rat urinary bladder.

2 The replacement of the bridging oxygen in the triphosphate chain of ATP ($pIC_{50} = 5.58$) with a methylene or imido group markedly increased the affinity (691 fold in IC_{50} values for β,γ-imidoATP, 15 fold for β,γ-methylene ATP), and the replacement of an ionized oxygen on the γ-phosphate with a sulphur (ATPγS) also led to increased affinity (5623 fold in IC_{50} values).

3 Modifications at N^6, N^1, and C-8 positions on the purine base usually reduced the affinity of ATP (a decrease of 2.8 fold in IC_{50} values for N^6-methylATP and 8.9 fold for 8-bromo ATP), while the attachment of an alkylthio group to the C-2 position greatly increased the affinity for P_{2X}-purinoceptors (from 3.5 to 98 fold increase in IC_{50} values).

4 Replacement of the 3′-hydroxyl group on the ribose with substituted amino or acylamino groups produced more potent P_{2X}-purinoceptor agonists (an increase of 447 fold in IC_{50} values for 3′-deoxy-3′-benzylamino ATP and 28 fold for 3′-deoxy-3′-(4-hydroxyphenylpropionyl)amino ATP.

5 Diadenosine polyphosphates (Ap[n]A) were also shown to displace the [^{3}H]-α,β-MeATP binding. The rank order of potency was Ap6A > Ap5A > Ap4A ≫ Ap3A ≫ Ap2A.

6 Suramin, PPADS, and reactive blue 2 could competitively displace the binding of [^{3}H]-α,β-MeATP to P_{2X}-purinoceptors, with pIC_{50} values of 6.26, 5.35, and 6.22, respectively.

Keywords: P_{2X}-purinoceptors; urinary bladder; [^{3}H]-α,β-methylene ATP; ATP derivatives; suramin; reactive blue 2; PPADS; radioligand binding assay

Introduction

Recent years have witnessed a significant expansion in the field of the physiological functions of extracellular purines. ATP, ADP, adenosine and some other purine derivatives were found to play the roles of neurotransmitter, co-transmitter, and neuromodulator in various types of tissues including both the central and peripheral nervous system (Burnstock, 1993). The receptors which mediate the effects of extracellular purines have been classified into two major types, i.e., P_1- and P_2-purinoceptors, mainly on the basis that the principal endogenous ligands were adenosine and ATP, respectively (Burnstock, 1978). P_2-purinoceptors have been further divided into P_{2X}-, P_{2Y}-, (Burnstock & Kennedy, 1985), P_{2T}-, P_{2Z}-purinoceptors (Gordon, 1986). Recently, some further subtypes of P_2-purinoceptors, P_{2U}-, P_{2N}-, have also been proposed (for review see Abbracchio *et al.*, 1993). The classification of P_2-purinoceptors was mainly based on the rank order of potencies of a series of purine compounds in evoking certain biological responses such as the contraction or relaxation of smooth muscles. Therefore, researchers in this field are concerned to develop more selective and more potent agonists for each subtype of P_2-purinoceptors, which would provide further evidence for the classification. Such work has been summarized by Cusack (1993).

P_{2X}-purinoceptors are mainly located on smooth muscle, where they mediate contraction. Recently, P_{2X}-purinoceptors have also been identified at neuro-neuronal synapses (Evans *et al.*, 1992; Edwards *et al.*, 1992; Silinsky *et al.*, 1992). In early as well as more recent investigations, rodent urinary bladder and vas deferens have been the two main organs used for pharmacological studies on P_{2X}-purinoceptors. Many studies have been carried out to explore the structure-activity relationships of purine compounds to elicit the P_{2X}-purinoceptor-mediated contraction (Lukacsko & Krell, 1982; Fedan *et al.*, 1982; 1986; Welford *et al.*, 1987; Howson *et al.*, 1988; Satchell, 1988). A lot of important information has been obtained from these studies. However, some factors, such as metabolic transformation and uptake of exogeneously applied ligands and the existence of multiple subtypes of receptors, may significantly influence the results from experiments on living tissues. Radioligand binding assay, on the other hand, deals directly with the interaction of a ligand and its receptors, offering an important tool to determine the structure-affinity relationship between ligands and receptors.

α,β-Methylene ATP is one of the most potent and relatively selective agonists for P_{2X}-purinoceptors. A few years ago, the tritiated form [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) was found to bind preferentially to P_{2X}-purinoceptors. The distribution of the binding sites is also in accordance with the distribution of P_{2X}-purinoceptors proven by pharmacological experiments (Bo & Burnstock, 1990; 1993b). Displacement experiments using several purine compounds showed that their rank order of potency in displacing the binding was in good agreement with that obtained from pharmacological experiments (Bo & Burnstock, 1990). Now several other laboratories have also begun to employ this radioligand to study P_{2X}-purinoceptors (Michel & Humphrey, 1993).

Recently, many new ATP derivaties with modifications of the triphosphate chain, purine base and the D-ribose have been synthesized in Dr. Jacobson's laboratories in NIH (Fischer *et al.*, 1993; Burnstock *et al.*, 1994). Functional studies like *in vitro* recording of smooth muscle responses and P_{2Y}-purinoceptor-promoted phospholipase C activity have shown that some of the compounds are more potent and more

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selective or specific for either P_{2X}- or P_{2Y}-purinoceptors (Fischer *et al.*, 1993; Burnstock *et al.*, 1994). In this study we used [^{3}H]-α,β-MeATP as a tool to examine the affinities of these new purine compounds and some obtained commercially for the P_{2X}-purinoceptors in rat urinary bladder. Several putative P_2-purinoceptor antagonists and a UTP derivative have also been tested. The results help to build up a profile of the structure-affinity relationship of the P_2-purinoceptor ligands with the receptors, which may provide some hints for the design of new P_{2X}-purinoceptor-selective compounds.

Methods

Male Wistar rats (200–250 g) were killed by asphyxiation with CO_2. Urinary bladders were removed immediately and placed in modified Krebs solution of the following composition (mM): NaCl 133, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, $NaHCO_3$ 16.3, NaH_2PO_4 1.4, glucose 7.7, (pH 7.4). They were trimmed free from surrounding adipose and connective tissues, minced and homogenized in 10 volume of ice-cold 50 mM Tris/HCl buffer containing 1 mM EGTA, 1 mM benzamidine hydrochloride, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.01% (w/v) bacitracin, and 0.002% (w/v) soybean trypsin inhibitors, pH 7.4 (buffer A), with a Polytron PT-3000 (Kinematica, Switzerland) twice in 15 s bursts at 30,000 r.p.m. The homogenate was centrifuged at 170 *g* for 5 min at 4°C. The supernatant was passed through double layers of nylon mesh and centrifuged at 105,000 *g* for 50 min at 4°C. The pellets were washed and suspended in buffer A and the protein content was determined by the method of Peterson (1977).

For saturation assays, serial concentrations of [^{3}H]-α,β-MeATP (from 0.55 to 160 nM) were incubated with bladder membrane preparations (about 30 μg protein per assay tube) in buffer A at 4°C for 120 min. At the end of the incubation the assay tubes were put on ice and the reaction mixture was rapidly filtered through a Whatman GF/B filter (presoaked in 20 mM disodium pyrophosphate solution) under vacuum. The filters were washed with 2 aliquots of 5 ml ice-cold 50 mM Tris/HCl buffer, pH 7.4, dried under an infrared lamp, and then the radioactivity trapped in the filters was measured in a Beckman SC6100I scintillation counter with an efficiency of about 56 to 61%. Non-specific binding was determined by including 100 μM β,γ-methylene ATP (β,γ-MeATP) in the incubation mixture. The assay was carried out in triplicate and each experiment was repeated five times.

Displacement experiments were carried out by incubating the membrane preparations (about 30 μg proteins per assay tube) with 10 nM [^{3}H]-α,β-MeATP and serial concentrations of an unlabelled ligand in buffer A at 4°C for 120 min (final volume 0.5 ml). The other procedures were the same as those for saturation assays.

Chemicals

[^{3}H]-α,β-MeATP was custom-synthesized by Amersham International plc. (Amersham, U.K.) with a specific activity of 19.2 Ci mmol^{-1} and chemical purity of 98–99%. The names, abbreviations, and sources of the non-labelled compounds used in this study are listed in Table 1. The chemical structures of some of the compounds are shown in Figure 1. Amongst the 50 compounds tested, all of them are putative agonists for P_2-purinoceptors except suramin, PPADS, and RB-2, which are regarded as P_2-purinoceptor antagonists.

Statistics

The data were expressed as mean ± standard error (s.e.mean). The data were analysed with the computer programme EBDA-LIGAND (Biosoft, Cambridge, UK) using non-linear curve fitting (Munson & Rodbard, 1980). The one or two binding site model was chosen based on the results of an *F* test on the weighted sum of squares. Student's *t* test was employed to compare the means of the pIC_{50} values. A *P* value less than 0.05 was considered as significant.

Results

The specific binding of [^{3}H]-α,β-MeATP to rat bladder membrane preparations was saturable and reversible. Computer-assisted non-linear least squares curve fitting of the binding data revealed two affinity sites, with K_d of 6.8 ± 1.9 nM and B_{max} of 9.4 ± 2.2 pmol mg^{-1} protein ($n = 5$) for the high-affinity sites, and K_d of 62.3 ± 18.8 nM and B_{max} of 47.5 ± 16.3 pmol mg^{-1} protein ($n = 5$) for the low-affinity sites. The specific binding in the displacement experiments was 6.9 ± 1.3 pmol mg^{-1} protein, and the non-specific binding (determined by 100 μM β,γ-MeATP) was about 10–15% of the total binding. The data from the displacement experiments have also been analysed with the computer programme LIGAND for one or two binding site fitting. The fittings for two binding sites were not significantly better than the fitting for one binding site. The main reason for the discrepancy with the two binding site fitting of the saturation assay may be that these compounds have no selectivity for the two affinity states of [^{3}H]-α,β-MeATP binding sites in rat bladder; therefore, near monophasic displacement curves were produced.

As shown in Table 2 and Figure 2 the potencies of the compounds tested in displacing the [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors in rat bladder vary significantly. In the first group of compounds with modifications on the triphosphate chain, the rank order of potency was the same as reported before (Burnstock & Kennedy, 1985), i.e., ATP > ADP ≫ AMP > AD, suggesting that the binding sites are P_2-purinoceptors. Replacement of an ionized oxygen in the triphosphate chain of ATP or the diphosphate group in ADP by a sulphur (ATPγS, ATPαS, and ADPβS) led to an increase of the affinities for P_{2X}-purinoceptors. ATPγS displayed the highest potency in displacing the binding of [^{3}H]-α,β-MeATP to P_{2X}-purinoceptors amongst the compounds tested in this study, which was about one thousand times higher than those of ADPβS and ATPαS ($P < 0.001$, Table 2, Figure 2a). However, the attachment of a big moiety to the phosphate at the γ-position like γ-(1[2-nitrophenyl]ethyl)ester (ATP-γ-E), did not significantly affect the affinity of ATP for the receptor. Some other moieties like ramidate (ApR, and AppR), morpholidate (ApM), and ribose (ADP-ribose), when attached to the end phosphate of AMP or ADP, abolished or significantly reduced (117 fold for ADP-ribose, $P < 0.001$ compared with ADP) their potencies in displacing the [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors. Attachment of a sulphate to the phosphate of AMP (AMP-SO_4) markedly increased its affinity for the receptors (407 fold in IC_{50} values, $P < 0.001$).

In the group of compounds with the phosphate-linking oxygen being replaced by methylene or imido, β,γ-imidoATP showed the highest affinity, followed by α,β-MeATP, β,γ-MeATP, and L-β,γ-MeATP (Table 2, Figure 2b). α,β-MeADP was almost without effect on the binding. Cyclization of the C-3′ and C-5′ of β,γ-MeATP to form cAMPPCP abolished the affinity of β,γ-MeATP for P_{2X}-purinoceptors.

Sodium tetraphosphate (PPPP) was almost as potent as ATP in displacing the binding, but sodium metatriphosphate (mPPP), a cyclic triphosphate structure, was about 113 fold less potent than the open chain triphosphate structure (sodium tripolyphosphate).

For the dinucleotides the pattern was quite evident: the longer the polyphosphate chain between the two adenosine groups, the more potent the compound in displacing the binding (Table 2, Figure 2c). The difference between the IC_{50} values of Ap6A and Ap2A is more than seven million times.

Table 1 The full names, abbreviations, and sources of the purine compounds and P_2-purinoceptor antagonists used in this study

Abbreviation	*Name*	*Source*
ATP	Adenosine 5′-triphosphate	Sigma
ADP	Adenosine 5′-diphosphate	Sigma
AMP	Adenosine 5′-monophosphate	Sigma
AD	Adenosine	Sigma
α,β-MeATP	Adenosine 5′-(α,β-methylene)triphosphate	Sigma
α,β-MeADP	Adenosine 5′-(α,β-methylene)diphosphate	Sigma
β,γ-MeATP	Adenosine 5′-(β,γ-methylene)triphosphate	Sigma
L-β,γ-MeATP	L-Adenosine 5′-(β,γ-methylene)triphosphate	Fisons
cAMPPCP	3′,5′-cyclic adenosine 5′-(β,γ-methylene)triphosphate	NIH
β,γ-ImidoATP	Adenosine 5′-(β,γ-imido)triphosphate	Sigma
PPPP	Sodium tetraphosphate	Sigma
mPPP	Sodium metatriphosphate	Sigma
ATPγS	Adenosine 5′-O-(3-thiotriphosphate)	Sigma
ATPαS	Adenosine 5′-O-(1-thiotriphosphate)	Sigma
ATP-γ-E	Adenosine 5′-triphosphate γ-(1[2-nitrophenyl]ethyl)ester	Sigma
ADPβS	Adenosine 5′-O-(2-thiodiphosphate)	Sigma
ApR	Adenosine 5′-monophosphoramidate	Sigma
AppR	Adenosine 5′-diphosphoramidate	Sigma
ApM	Adenosine 5′-monophosphomorpholidate	Sigma
AMP-SO_4	Adenosine 5′-monophosphosulphate	Sigma
ADP-ribose	Adenosine 5′-diphosphoribose	Sigma
Ap6A	P^1,P^6-diadenosine hexaphosphate	Sigma
Ap5A	P^1,P^5-diadenosine pentaphosphate	Sigma
Ap4A	P^1,P^4-diadenosine tetraphosphate	Sigma
Ap3A	P^1,P^3-diadenosine triphosphate	Sigma
Ap2A	P^1,P^2-diadenosine pyrophosphate	Sigma
N-O-ATP	Adenosine-N^1-oxide 5′-triphosphate	Sigma
AAHeATP	8-(6-aminohexyl)aminoadenosine 5′-triphosphate	NIH
8-BrATP	8-Bromoadenosine 5′-triphosphate	NIH
1,N^6-E-ATP	1,N^6-Ethenoadenosine 5′-triphosphate	Sigma
N^6-MeATP	N^6-Methyladenosine 5′-triphosphate	NIH
5-F-UTP	5-Fluorouridine 5′-triphosphate	NIH
2-MeSATP	2-Methylthioadenosine 5′-triphosphate	RBI
2-HeSATP	2-Hexylthioadenosine 5′-triphosphate	NIH
2-HexSADP	2-(5-Hexenyl)thioadenosine 5′-diphosphate	NIH
2-HexS-N^6-MeATP	2-(5-Hexenyl)thio-N^6-methyladenosine triphosphate	NIH
2-CyaHeSATP	2-(6-Cyanohexyl)thioadenosine 5′-triphosphate	NIH
2-CyaHeSAMP	2-(6-Cyanohexyl)thioadenosine 5′-monophosphate	NIH
2-cHeSATP	2-Cyclohexylthioadenosine 5′-triphosphate	NIH
2-NPESAMP	2-(2-*p*-Nitrophenylethyl)thioadenosine 5′-monophosphate	NIH
2′d-ATP	2′-deoxyadenosine 5′-triphosphate	Sigma
3′d-ATP	3′-deoxyadenosine 5′-triphosphate	Sigma
2′,3′dd-ATP	2′,3′-dideoxyadenosine 5′-triphosphate	Sigma
3′BH-NH-ATP	3′-deoxy-3′-(4-hydroxy-phenylpropionyl)aminoadenosine 5′-triphosphate	NIH
3′dBAATP	3′-deoxy-3′-benzylaminoadenosine 5′-triphosphate	NIH
2′,5′-ADP	Adenosine 2′,5′-diphosphate	Sigma
2′3′-iPIAMP	2′,3′-isopropylidene adenosine 5′-monophosphate	NIH
PPADS	Pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid	Lambrecht
RB-2	Reactive blue 2	Sigma
Suramin		Bayer

Sigma: Sigma Chemicals UK Ltd., Poole, UK; RBI: Research Biochemicals Incorporated, Natick, USA; Bayer: Bayer AG UK Ltd., Strawberry Hill, Newbury, UK; NIH: Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Disease, Bethesda, U.S.A.; Lambrecht: Prof. Lambrecht, University of Frankfurt, Germany.

Modifications to the purine base of ATP molecule usually lead to a reduction of ATP affinity for P_{2X}-purinoceptors except at the C-2 position (Table 2, Figure 2d). N-O-ATP, 1,N^6-E-ATP, N^6-MeATP, and 8-BrATP all showed reduced affinities relative to ATP (in IC_{50} values: 2.8 fold for N-O-ATP and N^6-MeATP, 4.5 fold for 1,N^6-E-ATP, and 8.9 fold for 8-BrATP), and their displacement curves were usually rather steep (Hill coefficients n_H from 1.67 to 2.15) (Figure 2) except 8-BrATP, which has a n_H of 0.7. AAHeATP has a (6-aminohexyl)amino group attached to the C-8 position, however, its potency was increased by 4.7 fold compared to ATP ($P<0.01$). The attachment of a thioether group to the C-2 position increased the affinity of ATP for P_{2X}-purinoceptors. The 2-hexylthio derivative 2-HeSATP possessed higher affinity than the 2-methylthio derivative 2-MeSATP (Table 2). The addition of a cyano group at the end of the hexyl chain of 2-HeSATP did not change its affinity. However, cyclization of the hexyl chain did reduce its potency by 6 times ($P<0.01$). The only UTP derivative tested in this study, 5-F-UTP, showed a 10 fold lower affinity for P_{2X}-purinoceptors than that of ATP.

Modifications to the ribose of ATP molecule also produced some potent ligands (Table 2, Figure 2e). Attachment of a large benzyl or phenylpropionyl moiety to the C-3′ position via an amino group (3′BH-NH-ATP and 3′dBAATP) significantly increased the affinity of ATP for P_{2X}-purinoceptors (28 and 447 fold, respectively, $P<0.01$). 3′d-ATP was about 4 fold more potent than 2′d-ATP in displacing [^{3}H]-α,β-MeATP binding ($P<0.05$), while the potency of 2′,3′dd-ATP fell in-between those of 2′d-ATP and 3′d-ATP and close to that of ATP. The addition of a phosphate group at the C-2′ position of AMP (2′,5′-ADP) significantly increased the affinity of AMP (9.3 fold in IC_{50} values).

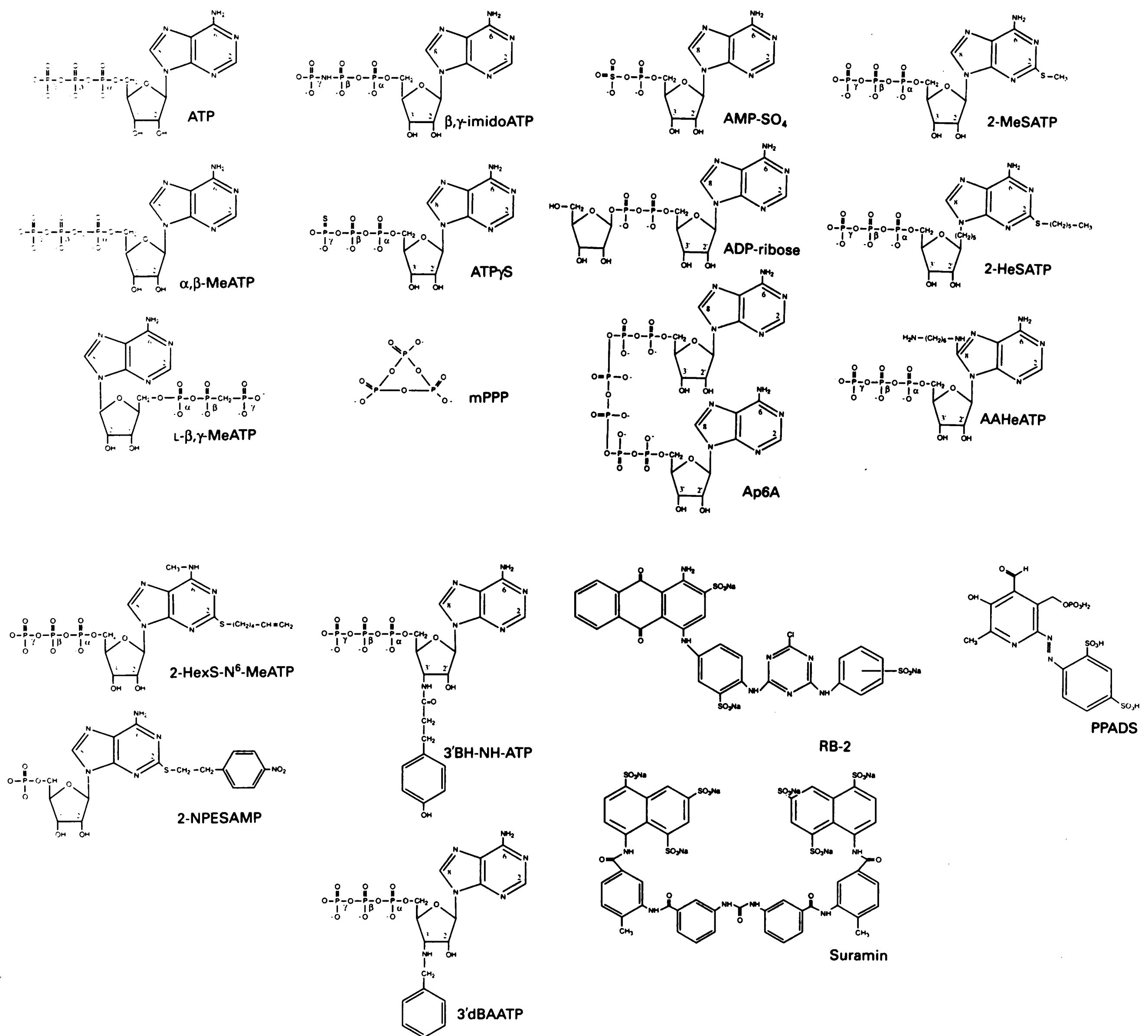

Figure 1 Chemical structures of the ATP derivatives and the P_2-purinoceptor antagonists.

Among the three putative P_2-purinoceptor antagonists, suramin and RB-2 were nearly equipotent in displacing the binding of [^{3}H]-α,β-MeATP. PPADS was about 7–8 times less potent in displacing the binding than suramin or RB-2 (Table 2). The displacement curve of RB-2 was rather steep (n_H = 1.46), while suramin and PPADS showed flatter sigmoid curves (n_H = 0.61 and 0.85, respectively) (Figure 2f).

Discussion

Studies on structure-activity relationship have shown that the triphosphate group in ATP molecule is responsible for the efficacy in activating P_{2X}-purinoceptors while the purine base and the D-ribose are associated with affinity (Lukacsko & Krell, 1982; Fedan *et al.*, 1986; Welford *et al.*, 1987; Howson *et al.*, 1988). Results from binding studies indicate that the triphosphate chain in the ATP molecule is also the key structure responsible for the affinity for P_{2X}-purinoceptors (Bo & Burnstock, 1993a). In this study, 46 newly synthesized or commercially available purine compounds with modifications at different positions of ATP, and one UTP analogue were tested, and remarkable differences in the affinities of the compounds for P_{2X}-purinoceptors have been observed.

First the compounds which are commonly used to distinguish P_1-, P_2-, P_{2X}-, and P_{2Y}-purinoceptors were examined again, since the experimental conditions in this study were different from those reported in a previous paper (Bo & Burnstock, 1990). Divalent cations can facilitate the degradation of ATP and its derivatives and influence the interaction of purine compounds with P_{2X}-purinoceptors (Bo & Burnstock, 1990); therefore, the incubation in this study was carried out in a media free of exogenous divalent cations and in the presence of 1 mM EGTA at 4°C. Such changes produced a significant increase in the potencies of the purine nucleotides for displacing the [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors. For α,β-MeATP, β,γ-MeATP, ATP and ADP, 10 to 12 fold increases in IC_{50} values were noted. The IC_{50} value of suramin was increased by 4 times, while the IC_{50} value of 2-MeSATP was increased by 127 times. The order of potency of these compounds is: α,β-MeATP > β,γ-MeATP > 2-MeSATP > ATP > ADP ≫ AMP ≫ AD, which indicates that the [^{3}H]-α,β-MeATP binding sites under these experimental conditions still conform to the criteria for P_{2X}-purinoceptors according to Burnstock & Kennedy (1985).

Table 2 The pIC_{50} values and Hill coefficients of purinoceptor ligands in displacing the binding of [^{3}H]-α,β-methylene ATP to rat urinary bladder membranes

Ligand	pIC_{50}	n_H
ATP and triphosphate modification		
ATP	5.58 ± 0.17	0.85 ± 0.11
ADP	5.32 ± 0.24	0.90 ± 0.13
AMP	4.09 ± 0.45	1.41 ± 0.21
AD	> 3.00	
α,β-MeATP	8.03 ± 0.11	0.97 ± 0.05
α,β-MeADP	> 3.00	
β,γ-MeATP	6.75 ± 0.04	1.07 ± 0.05
L-β,γ-MeATP	5.68 ± 0.15	0.67 ± 0.11
cAMPPCP	> 2.00	
β,γ-ImidoATP	8.42 ± 0.03	0.94 ± 0.20
PPPP	5.56 ± 0.09	0.87 ± 0.08
mPPP	2.98 ± 0.09	1.49 ± 0.65
ATPγS	9.33 ± 0.23	0.90 ± 0.04
ATPαS	6.39 ± 0.36	1.05 ± 0.04
ATP-γ-E	5.41 ± 0.05	1.19 ± 0.12
ADPβS	6.41 ± 0.12	0.90 ± 0.11
ApR	> 2.00	
AppR	> 2.00	
ApM	> 2.00	
AMP-SO4	6.70 ± 0.13	1.62 ± 0.07
ADP-ribose	3.25 ± 0.01	1.12 ± 0.02
Ap6A	8.88 ± 0.07	0.71 ± 0.06
Ap5A	8.05 ± 0.14	0.96 ± 0.03
Ap4A	7.57 ± 0.27	0.76 ± 0.06
Ap3A	5.16 ± 0.06	0.95 ± 0.10
Ap2A	> 2.00	
Base modification		
N-O-ATP	5.14 ± 0.07	1.76 ± 0.21
AAHeATP	6.25 ± 0.15	1.04 ± 0.09
8-BrATP	4.63 ± 0.21	0.70 ± 0.23
1,N^6-E-ATP	4.93 ± 0.03	1.67 ± 0.38
N^6-MeATP	5.14 ± 0.11	2.15 ± 0.13
5-F-UTP	4.60 ± 0.01	1.61 ± 0.09
2-MeSATP	6.33 ± 0.17	0.71 ± 0.12
2-HeSATP	7.57 ± 0.24	0.83 ± 0.04
2-HexSADP	6.01 ± 0.17	0.72 ± 0.16
2-HexS-N^6-MeATP	6.13 ± 0.15	0.72 ± 0.22
2-CyaHeSATP	7.24 ± 0.13	0.96 ± 0.10
2-CyaHeSAMP	4.34 ± 0.18	1.37 ± 0.16
2-cHeSATP	6.79 ± 0.23	1.06 ± 0.12
2-NPESAMP	5.42 ± 0.14	0.81 ± 0.11
Ribose modification		
2'd-ATP	5.28 ± 0.08	1.68 ± 0.22
3'd-ATP	5.92 ± 0.11	1.11 ± 0.13
2',3'dd-ATP	5.70 ± 0.14	1.14 ± 0.20
3'BH-NH-ATP	7.03 ± 0.23	0.76 ± 0.10
3'dBAATP	8.23 ± 0.19	1.12 ± 0.05
2'5'-ADP	5.06 ± 0.05	0.59 ± 0.05
2'3'-iPIAMP	5.33 ± 0.25	0.60 ± 0.09
Antagonists		
PPADS	5.35 ± 0.17	0.85 ± 0.08
RB-2	6.22 ± 0.04	1.46 ± 0.20
Suramin	6.26 ± 0.07	0.61 ± 0.09

It should also be mentioned that the Hill coefficients for the displacement curves of ATP and ADP have been reduced greatly (from 1.53 to 0.85 for ATP, and from 1.45 to 0.90 for ADP). The reason may be that the degradation of ATP and ADP had been inhibited under the experimental conditions used in this study so their effects at lower concentrations were revealed.

As has been reported by many researchers, the replacement of the bridging oxygen between the phosphates in ATP molecule with methylene or imido leads to an increase in the potency in activating P_{2X}-purinoceptors (Brown *et al.*, 1979; Lukacsko & Krell, 1982; Fedan *et al.*, 1982; 1986; Welford *et al.*, 1987; Howson *et al.*, 1988). α,β-MeATP, β,γ-MeATP, and β,γ-imidoATP all showed higher affinity for P_{2X}-purinoceptors than ATP. It was thought that α,β-MeADP would be more potent than ADP in displacing the binding of [^{3}H]-α,β-MeATP to P_{2X}-purinoceptors. α,β-MeADP, however, displayed almost no affinity for P_{2X}-purinoceptors. Results from pharmacological experiments also showed that α,β-MeADP produced lesser responses than ADP, and the characteristics of the responses are different from either those of ADP or those of α,β-MeATP (Fedan *et al.*, 1982), suggesting a different mechanism was involved. α,β-MeADP has been reported to be an inhibitor of ectonucleotidases (Bruns, 1980).

The L-enantiomer of β,γ-MeATP has been reported to be more selective for P_{2X}-purinoceptors and to be more resistant to dephosphorylation (Cusack *et al.*, 1987; Welford *et al.*, 1987). However, the potency of L-β,γ-MeATP in activating P_{2X}-purinoceptors was much less than β,γ-MeATP, and about equipotent with ATP (Welford *et al.*, 1987). This is in agreement with the binding results, which indicate that the IC_{50} value of L-β,γ-MeATP is 11 times higher than that of β,γ-MeATP ($P < 0.01$) and nearly equal to that of ATP ($P > 0.05$). Another modification of β,γ-MeATP, cyclization of C-2' and C-5' of the ribose (cAMPPCP), totally abolished the affinity of β,γ-MeATP for P_{2X}-purinoceptors. This phenomenon may be due to a significant change of conformation caused by the cyclization, thus, cAMPPCP may be unable to fit into the binding pocket. Pharmacological experiments proved that cAMPPCP activates neither P_{2X}- nor P_{2Y}-purinoceptors (Burnstock *et al.*, 1994).

The replacement of an ionized oxygen on the phosphate chain of ATP or ADP by a sulphur atom produces ATPγS, ATPαS, and ADPβS. These compounds showed enhanced affinities for P_{2X}-purinoceptors relative to ATP. It seems that the position of the sulphur on the phosphate chain is a determinant for the affinity. Thus, the γ-sulphur derivative, ATPγS, was a thousand times more potent than the α-sulphur derivative, ATPαS. Pharmacological experiments also showed that ATPγS was far more potent than ATP in eliciting contraction of guinea-pig bladder (Fedan *et al.*, 1982) and cat bladder (Theobald, 1992). However, this compound is weaker when compared to β,γ-MeATP and β,γ-imidoATP (Fedan *et al.*, 1982), which may be due to its non-selectivity for P_{2X}- and P_{2Y}-purinoceptors; the latter may mask the P_{2X}-purinoceptor-mediated responses in *in vitro* recording experiments. The attachment of a large group like a [1-(2-nitrophenyl)ethyl]ester at the γ-position did not affect the affinity of ATP for P_{2X}-purinoceptors. However, attachment of a large group like ribose, ramidate, or morpholidate to the terminal phosphate group of AMP or ADP significantly reduced the affinities (ADP ribose), or totally abolished their affinities (ApR, AppR, ApM) for P_{2X}-purinoceptors suggesting the property of the substituting group or atom is also a determinant for the affinity. These phenomena have also been observed in pharmacological experiments (Fedan *et al.*, 1982; 1986; Hoyle & Edwards, 1992). AMP-SO_4, a compound with a sulphate attached to the terminal phosphate of AMP, displayed a much higher affinity for P_{2X}-purinoceptors than ATP. However, in pharmacological experiments AMP-SO_4 was less potent than ATP in eliciting the contraction of guinea-pig vas deferens (Fedan *et al.*, 1982). It is unclear whether this discrepancy is due to a difference in the P_{2X}-purinoceptors in guinea-pig vas deferens versus rat urinary bladder, or because this compound is a partial agonist. The Hill coefficient of AMP-SO_4 displacement curve was significantly higher than unity which supports the assumption that another mechanism may be involved.

In a previous experiment we observed that sodium triphosphate displaces the binding of [^{3}H]-α,β-MeATP to rat bladder with a potency nearly equal to that of ATP (Bo & Burnstock, 1993a). In the present study a similar effect was observed in the displacement experiment with sodium tetraphosphate. Sodium metatriphosphate, a cyclic structure of triphosphate, was much less potent than triphosphate in

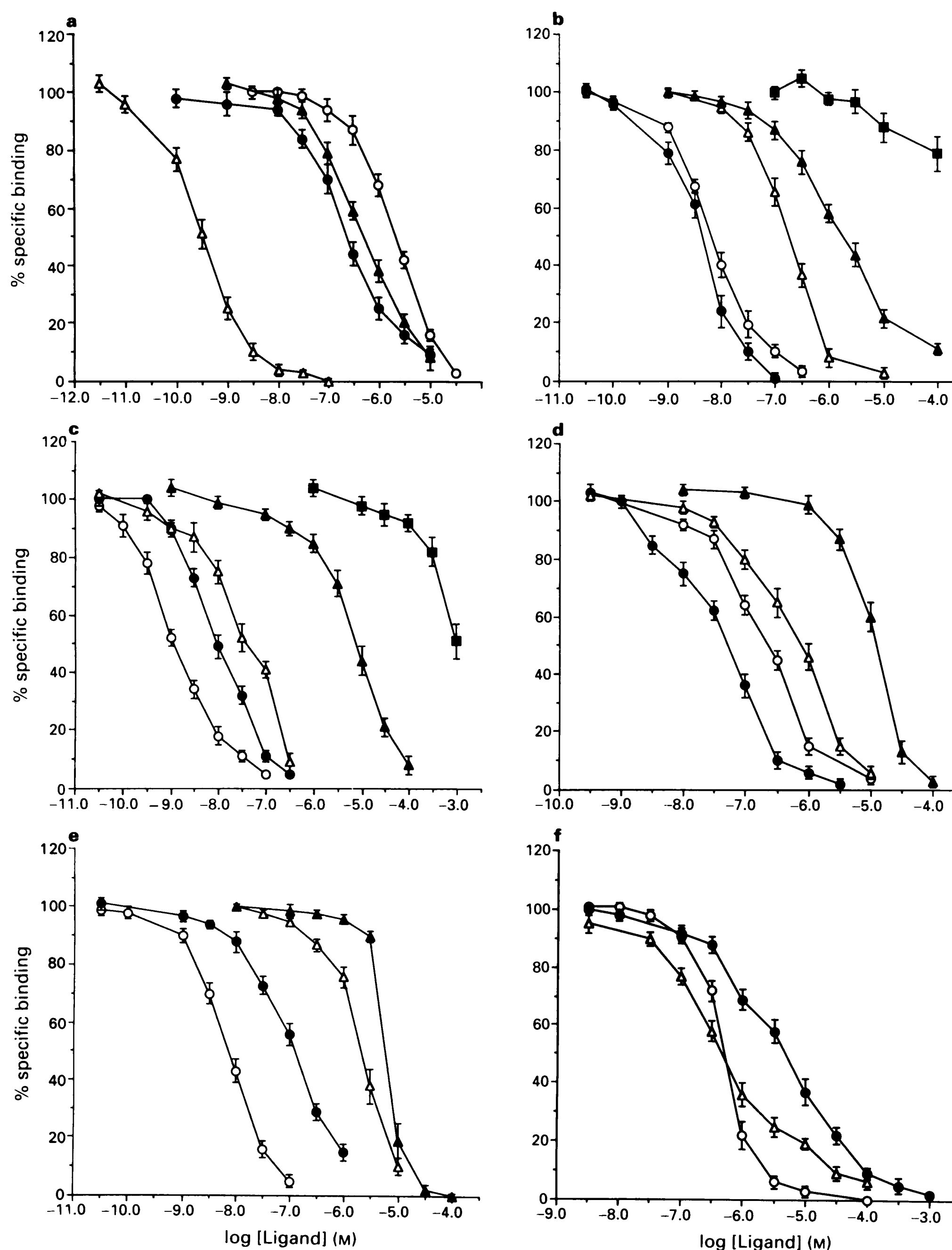

Figure 2 Displacement of [^{3}H]-α,β-methylene ATP binding to rat urinary bladder membranes by ATP derivatives and the P_2-purinoceptor antagonists. Rat urinary bladder membrane preparations were incubated with about 10 nM [^{3}H]-α,β-methylene ATP in the presence of the unlabelled ligand at 4°C for 2 h. (a) (○) ATP, (●) ADPβS, (▲) ATPαS, (△) ATPγS; (b) (○) α,β-MeATP. (●) β,γ-imidoATP, (△) β,γ-MeATP, (▲) L-β,γ-MeATP, (■) α,β-MeADP; (c) (○) Ap6A, (●) Ap5A, (△) Ap4A, (▲) Ap3A, (■) Ap2A; (d) (○) 2-MeSATP, (●) 2-CyaHeSATP, (△) 2-HexS-N^6-MeATP, (▲) 1,N^6-E-ATP; (e) (○) 3'dBAATP, (●) 3'BH-NH-ATP, (△) 3'd-ATP, (▲) 2'd-ATP; (f) (○) RB-2, (●) PPADS, (△) suramin. The points are the means with s.e.mean of five triplicated experiments.

displacing [^{3}H]-α,β-MeATP binding, suggesting that the triphosphate group in ATP molecule has to be a chain structure.

α,ω-Adenine dinucleotide polyphosphates have been found to exist in many mammalian tissues like hepatocytes (Rapaport & Zamecnik, 1976), adrenal medullary chromaffin granules (Rodriguez-del-Castillo *et al.*, 1988), and platelets (Lüthje & Ogilvie, 1983), which can be released upon stimulation. Their physiological function is unclear at present. Possible roles as neurotransmitters, cotransmitters, or trophic factors have been suggested (Hoyle, 1990). In pharmacological experiments these compounds have been reported to activate both P_{2X}- and P_{2Y}-purinoceptors (Stone, 1981; Busse *et al.*, 1988; Busshardt *et al.*, 1989; Stone & Paton, 1989; Hoyle *et al.*, 1989; Pintor *et al.*, 1991). The binding results from the present study demonstrated a clear order of affinities associated with the length of the polyphosphate chain linking the two adenosine moieties: Ap6A > Ap5A > Ap4A >> Ap3A >> Ap2A. This is in agreement with the results from pharmacological experiments which showed that Ap6A and Ap5A are as potent as α,β-MeATP in eliciting constrictions of guinea-pig vas deferens (MacKenzie *et al.*, 1988) and human urinary bladder (Hoyle *et al.*, 1989), and more potent than β,γ-MeATP in guinea-pig

urinary bladder (Stone, 1981). Therefore, it is possible that diadenine nucleotides may have a role as endogenous ligands for P_{2X}-purinoceptors as well as for their own specific receptors.

ATP derivatives with various modifications on the purine base have also been tested pharmacologically. These compounds, like N^6-phenyl ATP, N^6-MeATP, 8-BrATP, 8-azidoATP, N-O-ATP, 1,N^6-E-ATP, and formacyn-ATP, usually have diminished efficacy at P_{2X}-purinoceptors (Fedan *et al.*, 1982; 1986; Welford *et al.*, 1987; Howson *et al.*, 1988). In this study, N-O-ATP, 8-BrATP, and N^6-MeATP showed lower affinities for P_{2X}-purinoceptors than ATP, and their displacement curves were usually steep (with Hill coefficients significantly higher than unity except 8-BrATP), which suggests that their interaction with P_{2X}-purinoceptors may be different from those typical P_{2X}-purinoceptor agonists like α,β-MeATP and β,γ-MeATP. AAHeATP is an exception in this group of compounds. It did not show any agonist activity at the P_{2X}-purinoceptors in guinea-pig bladder and vas deferens, and rabbit saphenous artery (Burnstock *et al.*, 1994), yet its potency was 5 times higher than that of ATP. It is possible that this compound possesses some antagonistic property at P_{2X}-purinoceptors.

So far the only position on the purine base where modification can produce potent P_2-purinoceptor agonists is the C-2. 2-MeSATP has been known for a long time to be a potent P_{2Y}-purinoceptor agonist (Gough *et al.*, 1973; Satchell & Maguire, 1975; Burnstock *et al.*, 1983) although it can also activate P_{2X}-purinoceptors at a higher concentration (Howson *et al.*, 1988; Theobald, 1992). Further modifications on the C-2 position produced 2-HeSATP, 2-CyaHeSATP, 2-phenylethylthio ATP, and 2-(2-*p*-nitrophenylethyl)thio ATP, which did not show significantly increased efficacy on P_{2Y}-purinoceptors, while the potency on P_{2X}-purinoceptors have been greatly enhanced (Fischer *et al.*, 1993). Binding results from the present experiments showed that both 2-HeSATP and 2-CyaHeSATP had significantly higher potencies than 2-MeSATP ($P<0.01$). Cyclization of the hexyl group (2-cHeSATP) reduced the affinity of 2-HeSATP slightly. Addition of a methyl group at the N^6-position of 2-HexSATP (2-HexS-N^6-MeATP) reduced its affinity for P_{2X}-purinoceptors by 28 times. Pharmacological experiments showed that this compound had a higher selectivity for P_{2Y}-purinoceptors than 2-HexSATP (Fischer *et al.*, 1993). Taken together the observation that N^6-MeATP was selective for P_{2Y}-purinoceptors in guinea-pig taenia coli and inactive at P_{2X}-purinoceptors, suggested that the combination of the N^6 and C-2 modification may produce more selective and more potent agonists for P_{2Y}-purinoceptors (Fischer *et al.*, 1993).

5-F-UTP, like UTP ($pIC_{50}=4.67$, Bo & Burnstock, 1993a), was not very potent in displacing the [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors in rat bladder. However, in pharmacological experiments, it was very potent in inducing contraction of guinea-pig bladder detrusor (Burnstock *et al.*, 1994). Such response to 5-F-UTP may be mediated by the putatively termed P_{2U}-purinoceptors or 'pyrimidinoceptor' (Seifert & Schultz, 1989).

Modification on the ribose of ATP molecule also produced some compounds which displayed different potencies at P_{2X}-purinoceptors. Early pharmacological studies showed that the removal of the 2'-hydroxyl group of ATP led to a reduced potency for P_{2X}-purinoceptors, while removal of the 3'-hydroxyl group led to a slight increase in potency (Fedan *et al.*, 1982; 1986; Howson *et al.*, 1988). Such observations have been confirmed by the present experiments, showing 3'd-ATP had a potency about 4 fold higher than that of 2'd-ATP. Attachment of a (4-hydroxyphenylpropionyl)amino group at the C-3' position of 3'd-ATP (3'BH-NH-ATP) significantly enhanced the potency of 3'd-ATP in displacing the [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors. Another 3'd-ATP derivative which has a benzylamino group attached to the C'-3 position (3'dBAATP) was shown to be very potent in activating P_{2X}-purinoceptors in guinea-pig vas deferens and urinary bladder, while it was devoid of efficacy for P_{2Y}-purinoceptors (Burnstock *et al.*, 1994). Displacement experiments showed this compound had a very high affinity for P_{2X}-purinoceptors. Therefore, the replacement of the 3'-hydroxyl group with other specific groups does open a new area to search for more selective and more potent P_{2X}-purinoceptor agonists.

The development of specific antagonists for P_2-purinoceptors has not been so fruitful. For many years RB-2 has been used as an antagonist for P_{2Y}-purinoceptors (Kerr & Krantis, 1979) and suramin for P_{2X}-purinoceptors (Dunn & Blakeley, 1988). It was later found that their selectivity for either P_{2Y}- or P_{2X}-purinoceptor is not high (Choo, 1980; Fedan & Lamport, 1990; Hoyle *et al.*, 1990). PPADS has recently been reported as an antagonist for P_{2X}-purinoceptors (Lambrecht *et al.*, 1992). In this study, all three compounds could competitively displace the [^{3}H]-α,β-MeATP binding to rat bladder membranes (Figure 2f), however, the Hill coefficient of the RB-2 displacement curve was much higher than those of suramin and PPADS. The reason for such differences is unclear at present. It should be mentioned that in another similar experiment carried out on rabbit urinary bladder membranes PPADS was shown to interact with two affinity states of [^{3}H]-α,β-MeATP binding sites with K_i values of 22 nM and 48 μM, respectively (Ziganshin *et al.*, 1993), while in rat bladder membranes the one binding site model was preferred, which indicates that the properties of [^{3}H]-α,β-MeATP binding sites in rabbit and rat urinary bladder are somewhat different.

In this study, the order of potency of most of the ligands tested is in agreement with that obtained from pharmacological experiments. However, one question that may be raised is why the K_i values of many potent P_{2X}-purinoceptor agonists are in the nanomolar range, while their EC_{50} values in eliciting contractile responses in smooth muscle are in the micromolar range? Ideally, the pharmacology of the binding should correlate quantitatively with the pharmacology of receptor-mediated effects. In practice, such quantitative correlation is difficult to achieve, especially when agonists are used as radioligands (Ehlert *et al.*, 1984; Burt, 1985), because of some unknown sequence of events between agonist receptor occupation and measured pharmacological responses. A pharmacological response depends on transmitter-receptor-effector coupling, while the binding assay only deals with transmitter-receptor interaction. The influence of environmental factors also have important implications in the interpretation of agonist binding data. Pharmacological experiments on the P_{2X}-purinoceptor-mediated responses were carried out on intact tissues or cells in the presence of divalent cations at 37°C, while the binding assays were performed on separated membranes in Ca^{2+}-free media in the presence of EGTA at 4°C. For many P_{2X}-purinoceptor-active agonists, the situation is more complicated because accurate EC_{50} values are difficult to obtain due to their ability to desensitize the receptor. A proportion of P_{2X}-purinoceptors would be turned into an inactive state after exposure to these agonists, while the pharmacological responses can only be displayed by the receptors in their active state. The rapid degradation of some of the agonists by the ectonucleotidases abundantly present on the cell membranes may partially account for their low efficacy in activating P_{2X}-purinoceptors.

In conclusion, modifications on the triphosphate chain, adenine and ribose of ATP molecules can significantly change the affinity of ATP for P_{2X}-purinoceptors in rat urinary bladder. The replacement of the bridging oxygen in the triphosphate chain with a methylene or imido group markedly increased the affinity and selectivity, and the replacement of an ionized oxygen on the γ-phosphate with a sulphur also leads to increase of affinity. Modifications at N^6, N^1, and C-8 positions on the purine base usually reduces the affinity of ATP, while the attachment of an alkylthio group to the C-2 position greatly increases the affinity for P_{2X}-purinoceptors. Replacement of 3'-hydroxyl group on the

ribose with some specific groups produces more potent P_{2X}-purinoceptor agonists. Suramin, PPADS, and RB-2 can competitively displace the binding of [^{3}H]-α,β-MeATP for P_{2X}-purinoceptors, but their affinities are moderate.

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