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High- and low-affinity binding sites for [^{3}H]-α,β-methylene ATP in rat urinary bladder membranes

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1 The characteristics of [^{3}H]-α,β-methylene adenosine 5'-triphosphate ([^{3}H]-α,β-MeATP) binding to membrane preparations of rat urinary bladder detrusor were studied.

2 The rat bladder membrane preparation was obtained by multiple centrifugation. [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding to this preparation demonstrated that the muscarinic receptor density was 4.32 times higher than that in the homogenate. [^{3}H]-α,β-MeATP binding was increased 3.88 times.

3 Saturation analysis revealed that the rat bladder membrane contained a high density of [^{3}H]-α,β-MeATP binding sites, which could be divided into a high-affinity component (K_d = 8.1–8.9 nM) and a low-affinity component (K_d = 67.0–119.8 nM).

4 Magnesium ions inhibited the maximum binding in a concentration-dependent manner. The maximum high-affinity binding was reduced from 10.32 pmol mg^{-1} protein in magnesium-free buffer to 4.62 pmol mg^{-1} protein with 25 mM $MgCl_2$, while the maximum low-affinity binding was reduced from 58.84 pmol mg^{-1} protein to 14.24 pmol mg^{-1} protein. K_d values were not greatly affected.

5 The binding was a rapid reversible process. The association rate constants were 7.64 × 10^7 M^{-1} min^{-1} for high-affinity binding, and 7.31 × 10^6 M^{-1} min^{-1} for low-affinity binding. The dissociation rate constants were 0.2896 min^{-1} for high-affinity binding, and 0.6348 min^{-1} for the low-affinity binding.

6 Displacement experiments with unlabelled purinoceptor ligands confirmed that [^{3}H]-α,β-MeATP mainly binds to P_{2X}-purinoceptors. The potency order was: α,β-methylene ATP > β,γ-methylene ATP > suramin > ATP > ADP > 2-methylthio ATP ≫ adenosine.

7 The results indicate that [^{3}H]-α,β-MeATP is a radioligand for the P_{2X}-purinoceptor, which satisfies the basic criteria for use in radioligand binding assay.

Introduction

Amongst the growing literature on purinergic nerve transmission, there are many studies of mammalian urinary bladder. Excitatory purinergic responses have been reported in bladders of rabbit (Dean & Downie, 1978), guinea-pig (Burnstock *et al.*, 1978), rat (Burnstock *et al.*, 1972), mouse (Acevedo & Contreras, 1985), ferret and marmoset (Moss & Burnstock, 1985), and cat (Theobald, 1983). In human isolated bladder strips adenosine 5'-triphosphate (ATP) can induce concentration-dependent contractions (Husted *et al.*, 1983; Hoyle *et al.*, 1989). The atropine-resistant residuals of the electrical field stimulation-elicited contractions in the human bladder strips can be abolished following desensitization with α,β-methylene ATP (α,β-MeATP) (Hoyle *et al.*, 1989). The purinoceptors which mediate the exitatory mechanical responses of mammalian bladders to field stimulation, ATP and its analogues were classified as of the P_{2X}-subtype (Burnstock & Kennedy, 1985; Hourani *et al.*, 1985).

Radioligand binding assay and autoradiography of P_1-purinoceptors (A_1 and A_2 adenosine receptors) have been carried out for many years (Schwabe, 1985), and a series of radioligands are commercially available. In 1983 Levin *et al.* used [^{3}H]-ATP to label its binding sites in rabbit urinary bladder and reached the conclusion that the binding sites are not related to ATPase activity (Levin *et al.*, 1983). However, the rapid degradation of [^{3}H]-ATP limits its use in the characterization of P_2-purinoceptors. Specific binding sites for adenosine 5'-[α-[^{35}S]thio]triphosphate (ATPα[^{35}S]) have been observed on rat hepatocytes and purified liver plasma membranes (Keppens & De Wulf, 1986) and human liver plasma membranes (Keppens *et al.*, 1989). Competition experiments suggest that the binding sites are P_{2Y}-purinoceptors. The main disadvantage of this radioligand is the existence of the nonsaturable low-affinity binding site. Adenosine-5'-O-2-thio [^{35}S]-diphosphate ([^{35}S]-ADPβS) was reported as a radioligand for P_{2Y}-purinoceptors on turkey erythrocyte membrane (Cooper *et al.*, 1989). However, as was pointed out in that paper, [^{35}S]-ADPβS also has intracellular binding sites, which are unlikely to be P_{2Y}-purinoceptors. Therefore, this radioligand may not be suitable for the autoradiographic localization of P_{2Y}-purinoceptors. Recently we showed that [^{3}H]-α,β-MeATP is a radioligand for P_2-purinoceptors (preferentially for P_{2X}-purinoceptors) (Bo & Burnstock, 1989). In this paper we present a detailed study of the binding of [^{3}H]-α,β-MeATP to the membrane preparation of rat urinary bladder. The methods used in the first paper have been modified and extended.

Methods

Preparation of rat urinary bladder membranes

Male Wistar rats (200–250 g) were killed by cervical dislocation. Urinary bladders were removed immediately and the adipose tissue and serosa were trimmed off. The bladder was minced and homogenized in 3 ml 50 mM Tris-HCl buffer (pH 7.40, 20°C). The homogenate was centrifuged at 170 *g* for 4 min. The supernatant was drawn off and saved. The pellet was resuspended in 3 ml buffer, rehomogenized and recentrifuged as above. The supernatant was collected. The procedure was repeated four times. The supernatant was then centrifuged at 165,000 *g* for 1 h in a MSE Europa M-50 Ultracentrifuge. The supernatant was discarded and the pellet was suspended in 2 ml buffer for further dilution according to need. The harvest of crude membrane protein was 0.5 to 0.75 mg per bladder (70 to 76 mg wet weight). Protein concentration was determined by the method of Lowry *et al.* (1951).

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Estimation of membrane purity by [^{3}H]-quinuclidinyl benzilate ([^{3}H]-QNB) binding compared with [^{3}H]-α,β-MeATP binding

Two rat bladders were homogenized in 50 mM Tris-HCl buffer (containing 25 mM $MgCl_2$, pH 7.40). Two-thirds of the homogenate was centrifuged four times at 170*g* and the collected supernatant was then centrifuged at 165,000*g* for 1 h. The homogenate, supernatant and the pellet suspension from the ultracentrifugation were used for receptor binding. [^{3}H]-QNB (50 μl, final concentration ranging from 0.025 to 1.5 nM) was mixed with 50 μl Tris-HCl buffer and 400 μl homogenate, supernatant, or pellet suspension. To determine the non-specific binding, 50 μl atropine (10 μM) was added instead of 50 μl buffer. The incubation was carried out at 30°C for 30 min. The reaction was terminated by quick filtration through double layers of Whatman GF/C glass fibre filters. The filters were washed three times with 12 ml ice-cold buffer, soaked in 8 ml scintillation cocktail (OptiPhase MP, LKB) for 6 h, then the trapped radioactivity was measured in a Beckman LS 7500 scintillation counter with an efficiency of 46% to 50%.

In order to compare the binding of [^{3}H]-QNB and [^{3}H]-α,β-MeATP, parallel experiments were carried out on the binding of [^{3}H]-α,β-MeATP to the bladder homogenate, the supernatant and pellet suspension obtained from ultracentrifugation. The basic protocol is as described below with the exception that this experiment was done in the Tris-HCl buffer with 25 mM $MgCl_2$.

Saturation analysis and the effects of magnesium ions on [^{3}H]-α,β-MeATP binding

In the preliminary experiment we found that the magnesium ions in the incubation solution inhibited the [^{3}H]-α,β-MeATP binding. Therefore, parallel binding experiments were done in four kinds of incubation solutions containing either zero, 2 mM, 10 mM, or 25 mM $MgCl_2$. For one experiment the membrane preparations (in magnesium-free Tris-HCl buffer) from four rat bladders were mixed, and then divided into four 2 ml portions. Each portion was diluted to 13 ml with one of the four Tris-HCl buffer solutions containing zero, 2.95 mM, 14.8 mM, or 36.9 mM $MgCl_2$. Protein concentration was around 50 μg ml^{-1}. Stock solutions of [^{3}H]-α,β-MeATP and β,γ-methylene ATP (β,γ-MeATP) were prepared with magnesium-free Tris-HCl buffer. The tubes for total binding contained 50 μl [^{3}H]-α,β-MeATP (final concentration from 1.25 to 160 nM), 50 μl magnesium-free buffer and 400 μl membrane preparation. In the tubes for non-specific binding, 50 μl β,γ-MeATP (final concentration 100 μM) was added instead of 50 μl buffer. The incubation was carried out at 30°C for 15 min. The reaction was terminated by vacuum filtration through double layers of Whatman GF/C glass fibre filters. The filters were washed four times with 20 ml ice-cold buffer (with corresponding $MgCl_2$ concentration). The filters were put at the bottom of 20 ml scintillation vials and 8 ml of scintillation cocktail was added (OptiPhase MP, LKB). The samples were put aside for 6 h before measurements were taken. Data from 10 experiments were collected. Each experiment was carried out in duplicate.

Kinetics of [^{3}H]-α,β-MeATP binding

The plasma membrane was prepared in Tris-HCl buffer with 25 mM $MgCl_2$. For the measurement of the association rate, each tube contained 50 μl [^{3}H]-α,β-MeATP (final concentration 10 nM, in buffer with 25 mM $MgCl_2$), 50 μl buffer and 400 μl membrane preparation. The incubation was carried out at 30°C. The reaction was terminated at different times (from 5 s to 10 min) by rapid filtration under vacuum, followed by washing with 5 ml ice-cold buffer four times. For the measurement of dissociation rate the samples were prepared as above. They were incubated at 30°C for 15 min, and then 50 μl β,γ-MeATP (100 μM) was added to the mixture. The reaction was terminated at different times (from 15 s to 30 min).

The effects of purinoceptor ligands on the [^{3}H]-α,β-MeATP binding

Several compounds that have been shown by pharmacological experiments to be ligands for purinoceptors were chosen to displace the [^{3}H]-α,β-MeATP binding. [^{3}H]-α,β-MeATP (50 μl, final concentration 10 nM, in Tris-HCl buffer with 25 mM $MgCl_2$) was incubated with 400 μl membrane preparation in the presence of 50 μl non-labelled ligand. The concentration ranges of the non-labelled ligands were as follows: α,β-MeATP, 10^{-9}–10^{-4} M; β,γ-MeATP, 10^{-9}–10^{-4} M; suramin, 10^{-9}–10^{-4} M; 2-methylthio ATP (2-MeSATP), 10^{-9}–10^{-4} M; ATP, 10^{-9}–10^{-3} M; ADP, 10^{-9}–10^{-3} M; and adenosine, 10^{-9}–10^{-3} M. The incubation was carried out at 30°C for 15 min.

Other factors that could influence binding

The effect of pH on the [^{3}H]-α,β-MeATP binding was tested. [^{3}H]-α,β-MeATP (50 μl, 10 nM) was incubated with 400 μl membrane preparation and 50 μl buffer at 30°C for 15 min. The stock solution of [^{3}H]-α,β-MeATP and the membrane were prepared in four kinds of 50 mM Tris-HCl buffer (25 mM $MgCl_2$) with pH values of 6.0, 7.0, 7.5, 8.0, 9.0.

Some radioligands can bind to filters and can be partially displaced by a non-labelled ligand, which will produce false specific binding. The binding of [^{3}H]-α,β-MeATP to Whatman GF/C glass fibre filters was estimated. [^{3}H]-α,β-MeATP solutions (1.25–160 nM) with or without 100 μM β,γ-MeATP were filtered through double layers of GF/C filters, followed by washing with 5 ml ice-cold buffer four times.

Drugs

[^{3}H]-α,β-MeATP was synthesized by Amersham International plc. α,β-MeATP was tritiated by catalytic exchange. Specific activity was 27 Ci $mmol^{-1}$ with chemical purity of 98–99%. [^{3}H]-QNB with specific activity of 44 Ci $mmol^{-1}$ was purchased from the same company. α,β-MeATP, β,γ-MeATP, ATP, ADP, and adenosine were from Sigma Chemical Company Ltd. 2-MeSATP was from Research Biochemical Inc., U.S.A. Atropine sulphate was from Antigen Ltd. Suramin was a gift from ICI Pharmaceuticals, Alderly Park, Cheshire.

Results

The saturation analysis of [^{3}H]-QNB binding to rat bladder homogenate, the supernatant and pellet suspension from ultracentrifugation showed that the maximum specific binding was 112 ± 17 fmol mg^{-1} protein in the homogenate (K_d = 0.063 nM), 484 ± 37 fmol mg^{-1} protein in the pellet suspension (K_d = 0.054 nM). Only a trace amount of specific binding was observed in the supernatant. Thus, the muscarinic receptor density was increased 4.32 times by this membrane separation method. The maximum high-affinity specific binding of [^{3}H]-α,β-MeATP was 1235 ± 165 fmol mg^{-1} protein in the homogenate (K_d = 10.2 nM) and 4793 ± 385 fmol mg^{-1} protein in the pellet suspension (K_d = 8.5 nM). The density was therefore increased 3.88 times, which was a little lower than that for [^{3}H]-QNB binding. The reason for this may be that the supernatant also contained a small amount of specific [^{3}H]-α,β-MeATP binding (324 ± 57 fmol mg^{-1} protein).

The specific binding of [^{3}H]-α,β-MeATP to the rat bladder membrane preparation was saturable in all four concentrations of magnesium ion buffer tested. A typical binding curve (in the buffer with 25 mM $MgCl_2$) is shown in Figure 1. Scatchard analysis revealed the existence of two binding sites (Figure 2). The K_d values for the high-affinity binding varied between 8.1 and 8.9 nM, while for the low-affinity binding the

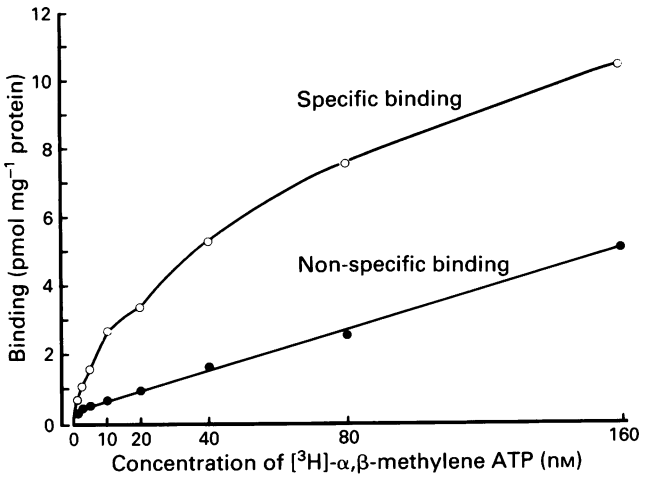

Figure 1 The saturation curve of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) binding to rat urinary bladder membrane preparation in 50 mM Tris-HCl buffer (pH 7.4) with 25 mM $MgCl_2$. Incubation was carried out at 30°C for 15 min. Non-specific binding (●) was determined by displacing the binding with 10 μM β,γ-MeATP. Each point represents the mean of 10 duplicated experiments.

range was 67.0 to 119.8 nM (Table 1). Magnesium ions had a significant inhibitory effect on the binding. The maximum high-affinity binding was 2.2 times higher in magnesium-free buffer than that in buffer with 25 mM $MgCl_2$, while the maximum low-affinity binding was 4.13 times higher in magnesium-free buffer than that in buffer with 25 mM $MgCl_2$. The K_d values for the high-affinity binding were slightly reduced with the increase of magnesium ion concentration in the buffer, but the K_d values for the low-affinity binding were reduced from 119.8 nM in the buffer with 2 mM $MgCl_2$ to 67 nM in the buffer with 25 mM $MgCl_2$. The non-specific binding was about 10% of the total binding at the lowest [^{3}H]-α,β-MeATP concentration and 50% at the highest [^{3}H]-α,β-MeATP concentration. Magnesium ions slightly increased the non-specific binding at lower [^{3}H]-α,β-MeATP concentrations (data not shown). The slopes of Hill plots showed that Hill coefficients (n_H) for all high- and low-affinity binding were around one (Table 1).

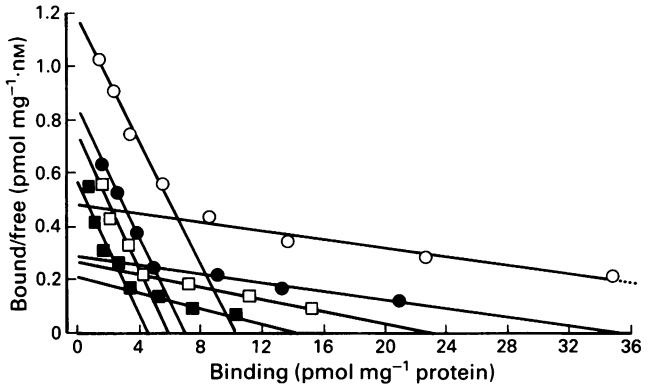

Figure 2 Scatchard plots of specific [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) binding to rat urinary bladder membrane preparation in Tris-HCl buffer (pH 7.4) with zero (○), 2 mM (●), 10 mM (□), or 25 mM (■) $MgCl_2$. Each point represents the mean of 10 duplicated experiments. The B_{max} and K_d values are shown in Table 1.

At 30°C the binding of [^{3}H]-α,β-MeATP to the bladder plasma membrane preparation was a rapid process, which reached equilibrium in 10 min (Figure 3). Kinetic analysis showed that the process was composed of two components: a high-affinity and a low-affinity part (Figure 3, inset). The association rate constant of the high-affinity component was $7.64 \times 10^7\ M^{-1}\ min^{-1}$, and that of the low-affinity component was $7.31 \times 10^6\ M^{-1}\ min^{-1}$.

The binding of [^{3}H]-MeATP to bladder membrane was a reversible reaction. After the binding of 10 nM [^{3}H]-α,β-MeATP had reached equilibrium, the addition of a high concentration of β,γ-MeATP (100 μM) quickly displaced the bound [^{3}H]-α,β-MeATP (Figure 4). The dissociation process was also composed of two components: the dissociation rate constant was $0.2896\ min^{-1}$ for the high-affinity binding, and $0.6348\ min^{-1}$ for the low-affinity binding (Figure 4, inset). The half-life of the [^{3}H]-α,β-MeATP-receptor complex was 2.31 min for the high-affinity binding sites and 1.09 min for the low-affinity binding sites. Thus the dissociation process was also very rapid at 30°C. The K_d values calculated from the kinetic experiment were 3.8 and 86.8 nM, which were in fair agreement with the K_d values obtained from the saturation experiment.

All the unlabelled ligands tested showed inhibitory effects on the binding of [^{3}H]-α,β-MeATP to different degrees (Figure 5). The competition of the binding by the agonists and antagonists was in a concentration-dependent manner. According to the IC_{50} values (Table 2) α,β-MeATP was the most potent agent to displace the binding with an IC_{50} value of 91 nM, which was more than 20 fold higher than the competitive actions of β,γ-MeATP and suramin. However, β,γ-

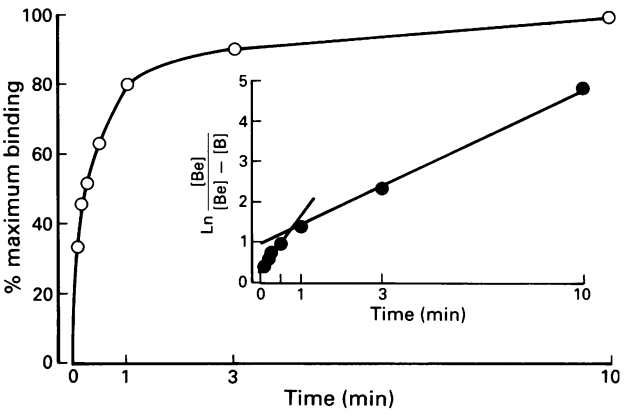

Figure 3 Association curve of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP, 10 nM) binding to rat urinary bladder membrane preparation at 30°C. Inset shows the time course after the original data are transformed with pseudo-first-order rate equation for the calculation of association rate constants. Data from seven experiments are collected. [Be]: concentration of receptor-radioligand complex at equilibrium, [B]: concentration of receptor-radioligand complex at the time the reaction was terminated.

Table 1 The maximum binding (B_{max}), K_d values and Hill coefficients (n_H) of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP) binding to rat urinary bladder membrane preparation at various magnesium ion concentrations (n = 7 to 10)

	High affinity			*Low affinity*		
	B_{max} (pmol mg^{-1} protein)	K_d (nM)	n_H	B_{max} (pmol mg^{-1} protein)	K_d (nM)	n_H
Mg^{2+}-free	10.32	8.9	0.99	58.84	112.7	1.00
2 mM $MgCl_2$	7.01	8.5	1.00	34.85	119.8	1.00
10 mM $MgCl_2$	5.97	8.3	1.00	23.68	91.7	1.01
25 mM $MgCl_2$	4.62	8.1	1.07	14.24	67.0	1.02

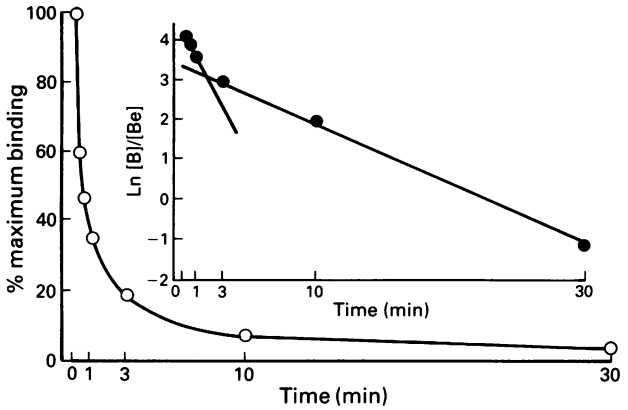

Figure 4 Dissociation curve of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP, 10 nM) binding to rat urinary bladder membrane preparation at 30°C. β,γ-MeATP (10 μM) was used to displace the bound [^{3}H]-α,β-MeATP after the binding reached equilibrium. Inset shows the time course after the original data are transformed with the equation for dissociation rate constant. Data from seven experiments are collected. [Be]: concentration of receptor-radioligand complex at equilibrium, [B]: concentration of receptor-radioligand complex at the time the reaction was terminated.

MeATP and suramin were 16 fold more potent than ATP. The IC_{50} values of ATP, ADP and 2-MeSATP were in the same order, but from the concentration-effect curve it was estimated that even at higher concentrations 2-MeSATP could only partially displace the specific binding, while the actions of ATP and ADP were complete. Adenosine was the least potent agent among the ligands tested, even at a concentration of 1 mM, it could displace only 13% of the specific binding.

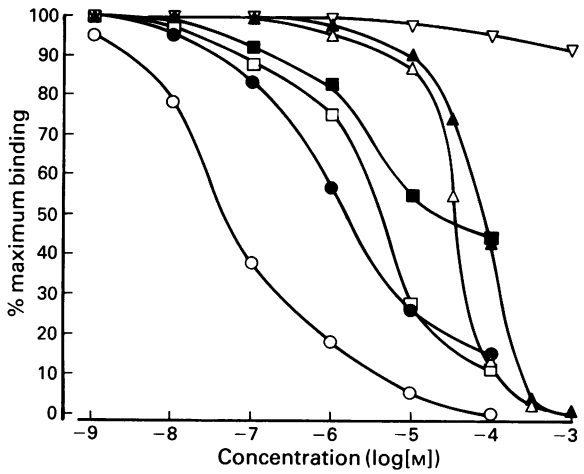

Figure 5 Displacement of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP, 10 nM) binding to rat urinary bladder membrane preparation by unlabelled ligands (in Tris-HCl buffer with 25 mM $MgCl_2$): (○), α,β-MeATP; (●) β,γ-MeATP; (□) suramin; (■) 2-methylthio ATP (2-MeSATP); (△) ATP; (▲) ADP; (▽) adenosine. Each point represents the mean from nine experiments.

Table 2 The IC_{50} values of purinoceptor active compounds to displace the binding of [^{3}H]-α,β-methylene ATP ([^{3}H]-α,β-MeATP, 10 nM) to rat bladder membrane preparation ($n = 9$)

Ligands	*IC_{50} (μM)*
α,β-MeATP	0.091
β,γ-MeATP	1.96
Suramin	2.20
ATP	32.6
ADP	55.0
2-MeSATP	59.6
Adenosine	⩾1000 (13%)*

* In parentheses is the percentage of the displaced binding at the maximum tested concentration of adenosine.
2-MeSATP = 2-methylthio ATP.

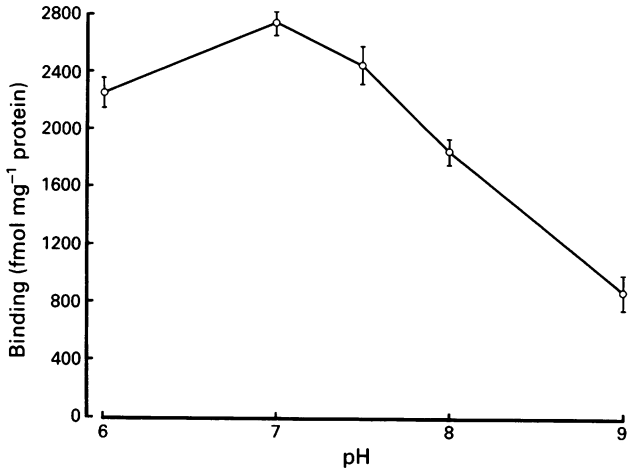

Figure 6 The effect of pH values of the incubation solution (50 mM Tris-HCl buffer with 25 mM $MgCl_2$) on the [^{3}H]-α,β-MeATP (10 nM) binding to rat urinary bladder membrane preparation. Data from six experiments are collected.

The effect of pH value on the [^{3}H]-α,β-MeATP binding is shown in Figure 6. The optimal pH value for the binding was around 7.0. It can be seen that an alkaline environment more profoundly inhibited the binding.

The binding of [^{3}H]-α,β-MeATP to Whatman GF/C glass fibre filters was very low, ranging from 0.14% (160 nM) to 0.22% (5 nM) of the total added radioactivity. No significant difference was observed between the binding with or without 100 μM β,γ-MeATP. Thus the interference of glass fibre filters on the [^{3}H]-α,β-MeATP binding can be excluded.

Discussion

Recently we reported in brief the discovery that [^{3}H]-α,β-MeATP can be used as a radioligand to label P_{2X}-purinoceptors (Bo & Burnstock, 1989). Because of the multiple functions of ATP in cell metabolism, we tried to develop a purified plasma membrane preparation to avoid the possibility of binding to sites on the intracellular organelles. It has been known that the separation of smooth muscle plasma membrane is more difficult than other kinds of tissues such as brain and liver (Batra, 1980). As to the urinary bladder, one successful separation of rabbit bladder membrane has been described (Batra, 1986). We modified the dextran-polyethylene glycol two-phase method (Brunette & Till, 1971) to purify the membrane of rat baldder. This method is very quick and does not need ultracentrifugation. The use of this kind of preparation had advantages over homogenate preparations for [^{3}H]-α,β-MeATP binding. However, the method is not as efficient in the separation of smooth muscle membranes as for L-cell membrane described in the original paper. In these experiments we used multiple centrifugation and harvested a purified plasma membrane. The [^{3}H]-QNB binding demonstrated that the muscarinic receptor density was increased by 4.32 times after the purification process, while the [^{3}H]-α,β-MeATP binding showed a similar increase. The parallel increase of binding sites of the two radioligands indicates that [^{3}H]-α,β-MeATP mainly binds to the proteins in the membrane and that the method is suitable to separate rat bladder membranes for use in radioligand binding assays.

In our first paper, saturation analysis showed only one [^{3}H]-α,β-MeATP binding site, with maximum binding of 3.52 pmol mg^{-1} protein and a K_d value of 58.2 nM in 50 mM Tris-HCl buffer with 25 mM $MgCl_2$. In fact, both the maximum binding and the K_d value were combined results of

two components. In the present experiments a purer membrane preparation was used and the concentration range of the radioligand was extended, so that the high- and low-affinity binding sites could be distinguished. The ratios of K_d values of the low- and high-affinity binding were around 8 to 14 fold. This phenomenon was common when agonists were used as the radioligands. In the radioligand binding assay of P_1-purinoceptors, [^{3}H]-(−)N^6-phenylisopropyladenosine ([^{3}H]-PIA) showed two binding sites in rat fat cell membrane, while [^{3}H]-^{5}N-ethylcarboxamidoadenosine ([^{3}H]-NECA) had two binding sites in human platelet membrane (Schwabe, 1985). In the experiments with [^{3}H]-ATP binding to rabbit bladder homogenate, two binding sites were also observed with K_d values of 20 nM and 45 nM for high- and low-affinity binding sites, respectively, which were not very far from our results.

Magnesium ions had profound effects on [^{3}H]-α,β-MeATP binding. With increase in magnesium ion concentration, both the high- and the low-affinity maximum binding were decreased. However, magnesium ions had only a slight effect on the K_d values for high-affinity binding, while the K_d values for the low-affinity binding showed larger increases at high magnesium concentration. The regulatory effects of magnesium ions and several other divalent ions such as manganese and calcium on the binding of P_1-purinoceptors have been known for many years (Goodman *et al.*, 1982; Hüttemann *et al.*, 1984). In the binding experiments at A_1-adenosine receptors, magnesium ions decreased the binding of [^{3}H]-N^6-cyclohexyladenosine ([^{3}H]-CHA) to rat fat cell membrane in the absence of guanine nucleotide; the binding was increased by magnesium ions in the presence of guanine nucleotide (Cooper *et al.*, 1984). Recently the study on P_{2Y}-purinoceptor binding with [^{35}S]-ADPβS indicated that without magnesium the high-affinity binding was almost completely lost (Cooper *et al.*, 1989). The binding of [^{3}H]-ATP to rabbit bladder homogenate also showed that a large proportion of the binding sites was magnesium-dependent (Levin *et al.*, 1983). The results from our experiment demonstrate that magnesium can modulate the P_{2X}-purinoceptor molecules and make the receptors lose their ability to bind to their ligands. The effect of guanine nucleotide on the [^{3}H]-α,β-MeATP binding has not yet been tested. Further investigation may reveal the relationship between P_{2X}-purinoceptor and G-protein, and may give more insight into the molecular mechanism of P_{2X}-purinoceptor action.

The existence of two binding sites for [^{3}H]-α,β-MeATP in rat bladder membrane was confirmed by the kinetic experiment. After the transformation of the original data with special equations, both the association and dissociation processes still demonstrated a curvilinear time-course, which could be separated into two components by curve-fitting. The K_d values derived from the kinetic experiment were similar to those from the saturation experiment, which implies that the binding may be a simple biomolecular reaction.

Both the association and dissociation processes were very rapid, but the [^{3}H]-α,β-MeATP-receptor complex lasted long enough to undergo vacuum filtration and the subsequent washing. The whole operation of filtration separation only took 12 s, so it can be estimated that the loss of the binding during separation was trivial.

The displacement experiment proved the specificity of [^{3}H]-α,β-MeATP binding to P_{2X}-purinoceptors. The potency order of the unlabelled ligands in displacing the [^{3}H]-α,β-MeATP binding is: α,β-MeATP > β,γ-MeATP > suramin > ATP > ADP > 2-MeSATP $\gg$ adenosine, which is in good agreement with the potency order of these ligands in their pharmacological actions on P_{2X}-purinoceptors (Burnstock & Kennedy, 1985). P_{2Y}-purinoceptor binding showed a different potency order: 2-MeSATP > ADPβS > ATP > ADP > App(NH)p > α,β-MeATP > β,γ-MeATP (Cooper *et al.*, 1989). This is also in accordance with the criteria for the classification of P_{2Y}-purinoceptors. It should be mentioned that the present displacement experiments were carried out at a radioligand concentration of 10 nM, which approximated to the K_d values for the high-affinity binding, thus the obtained potency order of the cold ligands probably represents their affinities to the high-affinity [^{3}H]-α,β-MeATP binding sites.

Suramin is a trypanocidal drug which was introduced into therapy in 1920. Recently it was reported that suramin could antagonize the response of mouse vas deferens to α,β-MeATP, and it was suggested as a specific antagonist for P_2-purinoceptors (Dunn & Blakeley, 1988). Our results confirm its affinity for P_{2X}-purinoceptors. However, pharmacological results from our laboratory show that suramin is also an antagonist of P_{2Y}-purinoceptors with an equal affinity (Hoyle *et al.*, 1990) so the action of suramin may not be specific.

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References

ACEVEDO, C.G. & CONTRERAS, R. (1985). Possible involvement of adenine nucleotides in the neurotransmission of the mouse urinary bladder. *Comp. Biochem. Physiol.*, **82C**, 357–361.

BATRA, S. (1980). Discussion summary: cell fractionation. In *Vascular Neuroeffector Mechanisms*. ed. Bevan, J.A., Godfraind, T., Maxwell, R.A. & Vanhoutte, P.M. pp. 9–12. New York: Raven Press.

BATRA, S. (1986). Use of ^{3}H-QNB in the isolation of plasma membrane from smooth muscle of the urinary bladder: effect of oxalate in calcium uptake by the membrane fractions. *Experientia*, **42**, 608–611.

BO, X. & BURNSTOCK, G. (1989). [^{3}H]-α,β-methylene ATP, a radioligand labelling P_2-purinoceptors. *J. Auton. Nerv. Syst.*, **28**, 85–88.

BRUNETTE, D.M. & TILL, J.E. (1971). A rapid method for the isolation of L-cell surface membranes using an aqueous two-phase polymer system. *J. Membrane Biol.*, **5**, 215–224.

BURNSTOCK, G., DUMSDAY, B. & SMYTHE, A. (1972). Atropine resistant excitation of the urinary bladder: the possibility of transmission via nerves releasing a purine nucleotide. *Br. J. Pharmacol.*, **44**, 451–461.

BURNSTOCK, G., COCKS, T., CROWE, R. & KASAKOV, L. (1978). Purinergic innervation of the guinea-pig urinary bladder. *Br. J. Pharmacol.*, **63**, 125–138.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **5**, 433–440.

COOPER, C.L., MORRIS, A.J. & HARDEN, T.K. (1989). Guanine nucleotide-sensitive interaction of a radiolabeled agonist with a phospholipase C-linked P_{2Y}-purinergic receptor. *J. Biol. Chem.*, **264**, 6202–6206.

COOPER, D.M.F., YEUNG, S-M.H., PEREZ-REYES, E. & FOSSOM, L.H. (1984). A central role for magnesium in the regulation of inhibitory adenosine receptors. In *Neurotransmitter Receptors, Mechanisms of Action and Regulation*. ed. Kito, K., Segawa, T., Kuriyama, K., Yamamura, H.I. & Olsen, R.W. pp. 17–30. New York: Plenum Press.

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

DEAN, D.M. & DOWNIE, J.W. (1978). Contribution of adrenergic and "purinergic" neurotransmission to contraction in rabbit detrusor. *J. Pharmacol. Exp. Ther.*, **207**, 431–445.

GOODMAN, R.R., COOPER, M.J., GAVISH, M. & SNYDER, S. (1982). Guanine nucleotide and cation regulation of the binding of [^{3}H]-cyclohexyladenosine and [^{3}H]-diethylphenylxanthine to adenosine A_1 receptors in brain membranes. *Mol. Pharmacol.*, **21**, 329–335.

HOURANI, S.M.O., WELFORD, L.A. & CUSACK, N.J. (1985). L-AMP-PCP, an ATP receptor agonist in guinea-pig bladder is inactive on taenia coli. *Eur. J. Pharmacol.*, **108**, 197–200.

HOYLE, C.H.V., CHAPPLE, C. & BURNSTOCK, G. (1989). Isolated human bladder: evidence for an adenine dinucleotide acting on P_{2X}-purinoceptors and for purinergic transmission. *Eur. J. Pharmacol.*, **174**, 115–118.

HOYLE, C.H.V., KNIGHT, G.E. & BURNSTOCK, G. (1990). Suramin antagonizes responses to P_2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.*, **99**, 617–621.

HUSTED, S., SJÖGREN, C. & ANDERSSON, K-E. (1983). Direct effects of adenosine and adenine nucleotides on isolated human urinary bladder and their influence on electrically induced contractions. *J. Urol.*, **130**, 392–398.

HÜTTEMANN, E., UKENA, D., LENSCHOW, V. & SCHWABE, U. (1984). R_a adenosine receptors in human platelets: characterization by 5'-N-ethylcarboxamido [^{3}H]adenosine binding in relation to adenylate cyclase activity. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **325**, 226–233.

KEPPENS, S. & DE WULF, H. (1986). Characterization of the liver P_2-purinoceptor involved in the activation of glycogen phosphorylase. *Biochem. J.*, **240**, 367–371.

KEPPENS, S., VANDEKERCKHOVE, A. & DE WULF, H. (1989). Characterization of purinoceptors present on human liver plasma membranes. *FEBS Lett.*, **248**, 137–140.

LEVIN, R.M., JACOBY, R. & WEIN, A.J. (1983). High-affinity, divalent ion-specific ^{3}H-ATP to homogenate derived from rabbit urinary bladder. *Mol. Pharmacol.*, **23**, 1–7.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265–275.

MOSS, H.E. & BURNSTOCK, G. (1985). A comparative study of electrical field stimulation of the guinea-pig, ferret and marmoset urinary bladder. *Eur. J. Pharmacol.*, **114**, 311–316.

SCHWABE, U. (1985). Use of radioligands in the identification, classification, and study of adenosine receptors. In *Methods Used in Adenosine Research*. ed. Paton, D.M. pp. 239–278. New York and London: Plenum Press.

THEOBALD, R.T. Jr. (1983). The effect of arylazido aminopropionyl ATP on atropine resistant contractions of the cat urinary bladder. *Life Sci.*, **32**, 2479–2484.

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