PPADS selectively antagonizes P_{2X}-purinoceptor-mediated responses in the rabbit urinary bladder

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1 Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS), an inhibitor of P_{2X}-purinoceptor-mediated responses in rabbit vas deferens, was investigated for its ability to antagonize contractions evoked by α,β -methylene ATP (α,β -MeATP), carbachol and electrical field stimulation in the rabbit urinary bladder detrusor muscle.

2 PPADS (1–30 μ M) caused concentration-dependent inhibition of contractions to the stable P_{2X}-purinoceptor agonist, α,β -MeATP, decreasing the maximum response to α,β -MeATP (30 μ M) at concentrations of 3–30 μ M. The pD₂ value for α,β -MeATP in the absence of PPADS was 6.52 ± 0.10 (8). In the presence of PPADS at concentrations of 1, 3, 10 and 30 μ M the negative log concentrations of α,β -MeATP that cause the same contractile response as the pD₂ value were significantly different from control, being respectively 6.17 ± 0.09 (8), 5.64 ± 0.12 (7), 5.15 ± 0.23 (7) and 4.78 ± 0.22 (5).

3 PPADS (1–30 μ M) caused concentration-dependent inhibition of contractions to stimulation of intramural purinergic nerves (1–32 Hz). There was a greater inhibition at lower frequencies (1–8 Hz) than at higher frequencies (16–32 Hz). PPADS, 30 μ M, did not produce significantly greater antagonism than 10 μ M.

4 PPADS (30 μ M) had no significant influence on the contractile potency of carbachol: the pD₂ values of carbachol in the absence and presence of PPADS were not significantly different being 6.42 ± 0.16 (5) and 6.33 ± 0.18 (5), respectively. However, PPADS caused a small, but significant, suppression of the maximal response of carbachol, reducing it by approximately 9%.

5 Radioligand binding studies carried out on rabbit bladder membranes with [³H]- α,β -methylene ATP ([³H]- α,β -MeATP) showed that PPADS concentration-dependently inhibited the binding of [³H]- α,β -MeATP to P_{2X}-purinoceptors, while the binding of [³H]-quinuclidinyl benzilate to muscarinic cholinergic receptors was not affected.

6 Thus, PPADS (1–30 μ M) antagonized responses mediated via P_{2X}-purinoceptors in the rabbit urinary bladder. It was selective for P₂-purinoceptor-mediated contractions rather than those mediated via muscarinic receptors. Binding studies demonstrated that the antagonistic effect of PPADS is via a direct interaction with P_{2X}-purinoceptors.

Keywords: PPADS; α,β -methylene ATP; [³H]- α,β -methylene ATP; P_{2X}-purinoceptors; carbachol; [³H]-quinuclidinyl benzilate; electrical field stimulation; rabbit urinary bladder

Introduction

It is widely accepted that there are two major types of purinoceptors, P₁ (mainly responsive to adenosine) and P₂ (mainly responsive to ATP) (Burnstock, 1978; 1990; Hoyle, 1992). They are primarily distinguished by different agonist and antagonist profiles, and by different transduction mechanisms (Burnstock & Buckley, 1985). P₂-purinoceptors have been further subdivided into P_{2X}- and P_{2Y}-receptors based largely on the rank order of agonist potency of adenosine triphosphate (ATP) analogues (Burnstock & Kennedy, 1985); in addition, several other subtypes of P₂-purinoceptors have been defined, including P_{2T}- and P_{2Z}-receptors (see Gordon, 1986; Hoyle, 1992). There are potent and selective antagonists of P₁-purinoceptors such as 8-phenyltheophylline and related compounds (Cooper & Londos, 1988; Williams, 1991), whereas a more definitive characterization of P₂-purinoceptor subtypes continues to be hindered by the lack of potent, selective and competitive antagonists. Suramin displays a fairly specific antagonistic activity at P₂-purinoceptors but only at concentrations higher than 10 μ M (Hoyle *et al.*, 1990). It does not discriminate between P_{2X}- and P_{2Y}-purinoceptors. Reactive blue 2 is only

useful within a narrow range of concentrations for blocking selectively P_{2Y}-purinoceptors, above which it has non-specific effects (Burnstock & Warland, 1987). Desensitization by α,β -methylene ATP (α,β -MeATP) is generally specific for P_{2X}-purinoceptors (Kasakov & Burnstock, 1983), and although it has been used successfully in many preparations, it is not a competitive antagonist. Arylazidoaminopropionyl ATP (ANAPP₃) forms a covalent attachment to the P_{2X}-purinoceptor following irradiation with ultraviolet light (Hogaboom *et al.*, 1980) and is also not competitive; further, the irreversible nature of its action limits its use in studying subtypes of the P₂-purinoceptor.

It has long been observed that at least two transmitters are involved in excitatory neuromuscular transmission in mammalian urinary bladder, and that acetylcholine and ATP are the predominant excitatory neurotransmitters mediating cholinergic, possibly via muscarinic M₃-receptors (Noronha-Blob *et al.*, 1991) and purinergic responses via P_{2X}-purinoceptors, respectively (Burnstock & Kennedy, 1985; Fujii, 1988; Hoyle *et al.*, 1990; Bo & Burnstock, 1992; Kishii *et al.*, 1992; Sneddon & McLees, 1992). The purinergic component of neurogenic contractions in mammalian urinary bladder strips can be blocked by the trypanocidal agent, suramin, albeit with low potency (Hoyle *et al.*, 1990), the

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photoaffinity ATP analogue, ANAPP₃ (Theobald, 1986) or by desensitization of P_{2X}-purinoceptors with α,β -MeATP (Kasakov & Burnstock, 1983; Hoyle & Burnstock, 1985; Moss & Burnstock, 1985; Hoyle *et al.*, 1989; Sneddon & McLees, 1992).

In the search for more potent and more selective antagonists of P₂-purinoceptors, Lambrecht *et al.* (1992) examined the activity of pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on contractile responses of the rabbit vas deferens to α,β -MeATP and to electrical field stimulation. They found PPADS (1–10 μ M) to be an apparently selective inhibitor of P_{2X}-purinoceptor-mediated responses. With these observations in mind, the current study was undertaken in order to evaluate the antagonistic properties of PPADS in another tissue of the same species with excitatory P_{2X}-purinoceptors, the rabbit urinary bladder (Fujii, 1988; Bo & Burnstock, 1992; Kishii *et al.*, 1992; Sneddon & McLees, 1992). PPADS was examined for interactions with exogenous α,β -MeATP and for effects on purinergic neuromuscular transmission. Radioligand binding assays were also carried out to study the interaction of PPADS with P_{2X}-purinoceptors as well as its selectivity for this type of receptor.

Methods

Organ bath experiments

Male New Zealand White rabbits (2–3 kg) were killed by an overdose of sodium pentobarbitone (Sagatal), which was injected via the marginal ear vein, and exsanguination. The urinary bladder was removed and longitudinal strips of the detrusor muscle, approximately 2 by 15 mm, were prepared. The strips were suspended vertically in 10 ml organ baths for isometric recording of mechanical activity. An initial load of 1 g was applied to the strips which were then allowed to equilibrate for at least 60 min. Electrical field stimulation was applied via two platinum-wire rings 2.5 mm in diameter, 10 mm apart, through which the strip was threaded. The modified Krebs solution for these experiments had the following composition (mM): NaCl 133, KCl 4.7, NaHCO₃ 16.4, MgSO₄ 0.6, NaH₂PO₄ 0.8, CaCl₂ 2.5 and glucose 7.7, and was gassed with 95% O₂/5% CO₂ (pH 7.3–7.4) and was maintained at 37 $\pm$ 1°C. In experiments with electrical field stimulation, atropine (0.3 μ M) was also included in the Krebs solution. Contractions were recorded by either a Dynamometer UF1 or Grass FT0C3 force-displacement transducer, and displayed on a Grass 79D ink-writing oscillograph. Electrical field stimulation was provided by a Grass S9 stimulator and applied at a given frequency (1–32 Hz) with a pulse width of 0.5 ms and supramaximal voltage, until a maximum contraction had been reached and the tone had declined by about a third. In the experiments with exogenous agonists, single doses of α,β -MeATP (0.1–30 μ M) or carbachol were added directly to the organ bath and were washed out after a maximum contraction had been reached. Intervals of at least 30 min, with a wash every 10 min, were allowed between consecutive applications of α,β -MeATP in order to avoid desensitization, otherwise at least 10 min were allowed for washout. Frequency-response and concentration-response relationships for α,β -MeATP were examined in preparations before and after incubation with PPADS (1–30 μ M, minimum 20 min). Carbachol (0.03–100 μ M) was tested in parallel on two preparations from each animal. After the first concentration-response curves were constructed on both preparations, one of them was incubated with 30 μ M PPADS (at least 20 min), and then the second concentration-response curves were constructed. Thus, the preparation to which PPADS had not been added, was used as a time-control for carbachol.

Binding experiments

The rabbits were killed as described above. Urinary bladders were removed immediately and placed in modified Krebs solution. They were trimmed free from surrounding adipose and connective tissues, minced and homogenized in 10 volumes of ice-cold 50 mM Tris/HCl buffer including various protease inhibitors (1 mM ethylene glycol-bis(β -aminoethyl ether) (EGTA), 1 mM benzamidine, 0.1 mM phenylmethylsulphonyl fluoride (PMSF), 0.01% bacitracin, and 0.002% soybean trypsin inhibitors, pH 7.4) with a Polytron PT-3000 homogenizer (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 the buffer for binding assay. The protein content in the membrane preparations was measured with the method described by Peterson (1977).

The displacement experiments on P_{2X}-purinoceptors were performed in a final volume of 0.5 ml including 10 nM [³H]- α,β -methylene ATP ([³H]- α,β -MeATP), 20 μ g membrane proteins, and PPADS in various concentrations. The incubation was carried out at 4°C for 2 h. The reaction was terminated by rapid filtration of the mixture through Whatman GF/B glass fibre filters presoaked in 20 mM disodium pyrophosphate under vacuum. The filters were washed with two aliquots of 5 ml ice-cold Tris/HCl buffer, dried under an infrared lamp, and then put into a mini-scintillation vial. To each vial, 4 ml OptiPhase 'Hi-Safe' II was added, and 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 with 100 μ M β,γ -methylene ATP. Each assay was carried out in triplicate and five assays were performed.

The basic procedure for the displacement experiment on muscarinic cholinergic receptors was the same as that for P_{2X}-purinoceptors. Final [³H]-quinuclidinyl benzilate ([³H]-QNB) concentration was 0.5 nM, the filters were presoaked in Tris/HCl buffer, and the non-specific binding was determined with 10 μ M atropine.

Drugs used

Atropine sulphate was obtained from Phoenix Pharmaceuticals Ltd. α,β -Methylene ATP lithium salt, carbamyl- β -methyl choline chloride (carbachol), β,γ -methylene ATP sodium salt, EGTA, benzamidine, PMSF, bacitracin, soybean trypsin inhibitors and Folin reagents were obtained from Sigma Chemical Co. Ltd. Sagatal was supplied by May and Baker. [³H]- 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%. [³H]-QNB with a specific activity of 44 Ci mmol⁻¹ was purchased from the same company. PPADS was synthesized in one of our laboratories.

Analysis of results

Means were compared by Student's paired and unpaired *t* tests. A probability of less than or equal to 0.05 was considered as significant. The data from binding studies were analysed with a computer programme EBDA-LIGAND (Munson & Rodbard, 1980). The models for one or two binding sites were chosen based on the results of the *F*-test on the weighted sum of squares. Data are presented as mean $\pm$ s.e.mean (*n*).

In the experiments with carbachol, responses were calculated as the percentage of the maximal carbachol contraction. The mean response of the preparations from each animal was calculated at each concentration of carbachol used. These mean responses for each animal were then subjected to a probit transformation (Bliss, 1935; Finney, 1971).

which converts the sigmoidal log-concentration curve to a straight line. Linear regression of the probit values against log concentration was then carried out in order to interpolate the log-concentration yielding a 50% response i.e. the pD₂ (Hoyle & Greenberg, 1988).

Results

In isolated strips of detrusor muscle, α,β -MeATP (0.1–30 μ M) caused concentration-dependent contractions with a maximum response of $55.1 \pm 3.2\%$ (9) relative to the maximum contraction evoked by KCl at a concentration of 240 mM (Figure 1). The pD₂ value (negative log EC₅₀) was 6.52 ± 0.10 (9). PPADS at 1–30 μ M caused concentration-dependent shifts to the right of the α,β -MeATP concentration-response curve. The negative log[α,β -MeATP], producing a contractile response in the presence of PPADS equivalent to that produced by the control pD₂ value was 6.17 ± 0.09 (8), 5.64 ± 0.12 (7) and 5.15 ± 0.23 (7) at PPADS concentrations of 1, 3 and 10 μ M, respectively. All these values were significantly different both from control and from each other ($P < 0.05$, paired and unpaired *t* tests). However, the $-\log[\alpha,\beta$ -MeATP] value of 4.78 ± 0.22 (5) obtained at 30 μ M PPADS was not significantly different from that at 10 μ M.

In the presence of atropine (0.3 μ M), electrical field stimulation produced frequency-dependent contractions of the rabbit isolated detrusor muscle with maximum at a frequency of 8 Hz (Figure 2). PPADS (1–30 μ M) caused concentration-dependent inhibition of contractions evoked by electrical field stimulation. At a concentration of 1 μ M, PPADS inhibited contractile responses only at lower frequencies (2–8 Hz). At a concentration of 3 μ M, the significant inhibition of contractile responses to electrical field stimulation by PPADS extended up to 16 Hz. PPADS at 10 and 30 μ M significantly inhibited neurogenic contractions of detrusor muscle at all frequencies (1–32 Hz). However, there

was no significant difference between the inhibitions produced by PPADS at these two concentrations. PPADS at all applied concentrations (1–30 μ M) caused a greater inhibition at lower stimulation frequencies (1–8 Hz) than at higher stimulation frequencies (16 and 32 Hz).

Carbachol (0.03–100 μ M) caused concentration-dependent contractions of the detrusor muscle with a maximum contraction of $130.8 \pm 5.2\%$ (5) relative to that evoked by KCl

Figure 2 Rabbit urinary bladder, isolated detrusor muscle. Frequency-response relationships. Responses (ordinate axis) are expressed as a percentage of contraction evoked by electrical field stimulation at a frequency of 16 Hz in controls. Frequency-response curves are shown in the absence (∇ , $n = 9$) and presence of PPADS at concentrations of 1 μ M ($\circ$, $n = 8$), 3 μ M ($\bullet$, $n = 8$), 10 μ M ($\square$, $n = 7$) and 30 μ M ($\blacksquare$, $n = 7$). Atropine (0.3 μ M) was present throughout. Points show mean $\pm$ s.e.mean. The abscissae are on a logarithmic scale.

Figure 1 Rabbit urinary bladder, isolated detrusor muscle: concentration-response relationships for α,β -methylene ATP (α,β -MeATP) in the presence and absence of PPADS. Responses (ordinate axis) are expressed as percentage relative to the maximum contraction to 240 mM KCl. Concentration-response curves are shown for α,β -MeATP in the absence (∇ , $n = 9$) and presence of PPADS at concentrations of 1 μ M ($\circ$, $n = 8$), 3 μ M ($\bullet$, $n = 8$), 10 μ M ($\square$, $n = 8$) and 30 μ M ($\blacksquare$, $n = 5$). The points represent the mean $\pm$ s.e.mean.

Figure 3 Rabbit urinary bladder, isolated detrusor muscle: concentration-response curves for carbachol in the absence (∇ , $n = 5$) and in the presence of PPADS at 30 μ M ($\blacktriangledown$, $n = 5$). Points show the mean $\pm$ s.e.mean. Responses are expressed relative to the contracture produced by a standard concentration of KCl (240 mM). * $P < 0.05$.

Figure 4 Displacement of [3 H]- α,β -methylene ATP ([3 H]- α,β -MeATP) (○) and [3 H]-quinuclidinylbenzilate ([3 H]-QNB) (●) binding to rabbit urinary bladder membranes by PPADS. Membrane preparations of rabbit urinary bladder were incubated with 10 nM [3 H]- α,β -MeATP or 0.5 nM [3 H]-QNB in the presence of various concentrations of PPADS. Non-specific binding was determined with 100 μ M β,γ -methylene ATP or 10 μ M atropine respectively. Data shown are mean $\pm$ s.e. mean from five triplicate experiments.

at a concentration of 240 mM. In the time-control preparations, there was no significant difference between the first and the second pD_2 values for carbachol being 6.38 ± 0.13 (5) and 6.36 ± 0.11 (5), respectively. PPADS (30 μ M) did not significantly affect the pD_2 values of carbachol which were 6.42 ± 0.14 (5) and 6.37 ± 0.18 (5) in the absence and presence of PPADS, respectively. However, there was a small but significant decrease of the maximum carbachol contraction in the presence of PPADS which was $119.6 \pm 5.6\%$ (5) ($P < 0.05$, paired t test) relative to that evoked by 240 mM KCl (Figure 3).

PPADS at any concentration did not significantly change the basal tone or spontaneous activity of the rabbit urinary bladder. The effect of PPADS (30 μ M) did not appear to be readily reversible. After prolonged wash-out (> 2 h) the responses to electrical field stimulation and α,β -MeATP recovered only by 15–20%.

The displacement of [3 H]- α,β -MeATP binding to rabbit bladder membrane preparations by PPADS was concentration-dependent (Figure 4). Curvilinear fitting of the data revealed that PPADS interacted with the two affinity states of the P_{2X} -purinoceptor for [3 H]- α,β -MeATP with K_i values of $2.2 \pm 0.3 \times 10^{-8}$ M (5) and $4.8 \pm 1.1 \times 10^{-5}$ M (5), respectively. PPADS showed no obvious effect on the binding of [3 H]-QNB to muscarinic cholinergic receptors in the rabbit bladder membranes (Figure 4).

Discussion

The results show that PPADS had an antagonistic effect on P_{2X} -purinoceptors in the rabbit urinary bladder detrusor muscle. The action of PPADS seemed to be selective for the P_{2X} -purinoceptors, since there was no change in the potency of carbachol for contracting the bladder detrusor muscle via muscarinic receptors, and no displacement of specific [3 H]-QNB binding to muscarinic receptors, although there was a small decrease in the efficacy of carbachol to contract the detrusor muscle. These results are consistent with those described for the action of PPADS in rabbit vas deferens (Lambrecht *et al.*, 1992).

PPADS at a concentration of 1 μ M, significantly affected the concentration-response relationship to α,β -MeATP, but caused significant inhibition of the purinergic contractions evoked by electrical field stimulation only at 2–8 Hz. At

concentrations of 3 and 10 μ M, PPADS caused a greater antagonism of P_{2X} -purinoceptor-mediated responses evoked either by exogenous α,β -MeATP or by purinergic nerve stimulation. It seems that PPADS had a maximum antagonistic effect on P_{2X} -purinoceptors in bladder detrusor muscle at a concentration around 10 μ M, since there was no significant difference between the inhibition caused by PPADS at 10 and 30 μ M in experiments with either α,β -MeATP or electrical field stimulation.

Mammalian urinary bladder detrusor muscle is provided with parasympathetic excitatory innervation and acetylcholine is a neurotransmitter in these nerves. However, it is well known, that the muscarinic antagonist atropine is unable to abolish the excitatory response induced by nerve stimulation (Langley & Anderson, 1895; Ursillo, 1961; Longhurst *et al.*, 1984). It has been suggested that the acetylcholine-induced contraction of bladder muscle is mediated via muscarinic M_3 -receptors (Noronha-Blob *et al.*, 1991) and that the atropine-resistant component is purinergic with ATP as the principle neurotransmitter (Burnstock *et al.*, 1978; Hoyle & Burnstock, 1985; Fujii, 1988). The purinoceptors that mediate the excitatory mechanical responses of rabbit (and other mammalian species) bladder to field stimulation and to exogenous ATP and related agonists were classified as of the P_{2X} -subtype, based on the relative potencies of ATP and its analogues (α,β -MeATP $>$ β,γ -methylene ATP $>$ ATP) (Burnstock & Kennedy, 1985; Kishii *et al.*, 1992; Sneddon & McLees, 1992), on the selective binding of [3 H]- α,β -MeATP to rabbit bladder smooth muscle (Bo & Burnstock, 1992), on selective desensitization by α,β -MeATP of ATP-mediated responses (Chen, 1990; Sneddon & McLees, 1992), and on the inability of the putative P_{2Y} -purinoceptor antagonist, reactive blue 2, to antagonize ATP-induced contraction (Kishii *et al.*, 1992).

In our experiments with electrical field stimulation, atropine (0.3 μ M) was present in the Krebs solution to exclude cholinergic transmission. Thus, inhibition by PPADS of neurogenic contractions was due to antagonism of the purinergic component. Lambrecht *et al.* (1992) have reported that PPADS is an antagonist of P_{2X} -purinoceptor-mediated responses in the rabbit vas deferens. At concentrations of 30 and 10 μ M, it abolished contractions of the rabbit vas deferens evoked by electrical field stimulation in the presence of prazosin (1 μ M) and to exogenous α,β -MeATP (up to 100 μ M), respectively. In the rabbit urinary bladder, PPADS (up to 30 μ M) did not abolish the neurogenic contractions or the contractile responses to α,β -MeATP. This implies that there may be some differences between the P_{2X} -purinoceptors in rabbit vas deferens and urinary bladder, as has already been noted amongst different species (Hoyle & Burnstock, 1991; Bo & Burnstock, 1992; Hoyle, 1992; Theobald, 1992). In addition, the resistance of a significant level of the contraction to α,β -MeATP and electrical field stimulation, shown by PPADS, in rabbit bladder, could possibly be due to the presence of multiple excitatory P_2 -purinoceptor subtypes (Theobald, 1992), at least one of which is resistant to PPADS. Thus, further experiments with PPADS on other tissues exhibiting clear-cut P_2 -purinoceptor subtype-mediated responses could be useful for a more complete characterization of the P_2 -purinoceptors in rabbit bladder.

[3 H]- α,β -MeATP has been proved to bind preferentially to P_{2X} -purinoceptors in radioligand binding assays and autoradiographic studies (Bo & Burnstock, 1989; 1990). In this study, PPADS was shown to displace competitively the binding of [3 H]- α,β -MeATP to rabbit bladder membranes, which indicates that PPADS acts directly on P_{2X} -purinoceptors. In the rabbit bladder membrane preparations it has been demonstrated that [3 H]- α,β -MeATP binds to two affinity states of P_{2X} -purinoceptors (Bo & Burnstock, 1992). Computer-assisted curve-fitting of the data revealed that PPADS displaced the [3 H]- α,β -MeATP binding to rabbit bladder membranes also from two affinity states, thus confirming the observation from the saturation assays. PPADS showed no

effects on [³H]-QNB binding to muscarinic cholinceptors in rabbit bladder membranes in the range of concentrations tested. Therefore, the observed slight inhibition of cholinergic responses by PPADS was via mechanisms other than a direct action on muscarinic cholinceptors.

In conclusion, PPADS (1–30 µM) blocks P_{2X}-purinoceptor-mediated responses in the rabbit urinary bladder. Such an action of PPADS is due to its direct antagonism of P_{2X}-purinoceptors. If further studies clarify the subtype-selectivity, this compound might become the starting point for the development of a new generation of selective purinoceptor antagonists.

The authors thank the Wellcome Trust (A.U.Z.), the British Heart Foundation (X.B.), the Fonds der Chemischen Industrie (Germany) (G.L., E.M.) and the Deutsche Forschungsgemeinschaft (H.G.B., SFB 169) for financial support.

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(Received April 28, 1993)

Revised July 8, 1993

Accepted July 26, 1993