British Journal of Pharmacology (1995) 115, 1509-1517

Inhibitory action of PPADS on relaxant responses to adenine nucleotides or electrical field stimulation in guinea-pig taenia coli and rat duodenum

*†Ursula Windscheif, †Otmar Pfaff, *#Airat U. Ziganshin, *Charles H.V. Hoyle, **Hans G. Bäumert, †Ernst Mutschler, *Geoffrey Burnstock & [1,]†Günter Lambrecht

*Department of Anatomy and Developmental Biology and Centre for Neuroscience, University College London, Gower Street, London WC1E 6BT; Departments of †Pharmacology and **Biochemistry, Biocentre Niederursel, University of Frankfurt, Marie-Curie-Straße 9, D-60439 Frankfurt, Germany and #Kazan Medical Institute, 49 Butlerov Street, Kazan, 420012, Russia

1 The effect of pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) on the relaxant response to adenine nucleotides was examined in the carbachol-contracted guinea-pig taenia coli and rat duodenum, two tissues possessing P_{2y}-purinoceptors. In addition, in the taenia coli PPADS was investigated for its effect on relaxations evoked by adenosine, noradrenaline and electrical field stimulation. In order to assess the selectivity of PPADS between P_2-purinoceptor blockade and ecto-nucleotidase activity, its influence on ATP degradation was studied in guinea-pig taenia coli.

2 The resulting rank order of potency for the adenine nucleotides in guinea-pig taenia coli was: 2-methylthio ATP > > ATP > α,β-methylene ATP with the respective pD_2-values 7.96 ± 0.08 ($n=23$), 6.27 ± 0.12 ($n=21$) and 5.88 ± 0.04 ($n=24$).

3 In guinea-pig taenia coli, PPADS (10-100 μM) caused a consistent dextral shift of the concentration-response curve (CRC) of 2-methylthio ATP and ATP resulting in a biphasic Schild plot. A substantial shift was only observed at 100 μM PPADS, the respective pA_2-values at this particular concentration were 5.26 ± 0.16 ($n=5$) and 5.15 ± 0.13 ($n=6$). Lower concentrations of PPADS (3-30 μM) antagonized the relaxant effects to α,β-methylene ATP in a surmountable manner. An extensive shift of the CRC was produced only by 30 μM PPADS ($pA_2 = 5.97 \pm 0.08$, $n=6$), and the Schild plot was again biphasic.

4 The relaxant responses to electrical field stimulation (80 V, 0.3 ms, 5 s, 0.5-16 Hz) in guinea-pig taenia coli were concentration-dependently inhibited by PPADS (10-100 μM).

5 In guinea-pig taenia coli, the potency of ATP in inducing relaxation appeared to be independent of its rate of degradation by ecto-nucleotidases, since the K_m-value (366 μM) obtained in the enzyme assay was much higher than the functional EC_{50}-value (0.45 μM) of ATP. PPADS (3-100 μM) was only weakly active in inhibiting ecto-nucleotidase activity leaving a residual activity of $81.8 \pm 5.1\%$ at 100 μM. Enzyme inhibition by PPADS was concentration-independent and non-competitive.

6 In rat duodenum, the rank order of potency was: 2-methylthio ATP > ATP > > α,β-methylene ATP, the respective pD_2-values being 6.98 ± 0.04 ($n=76$), 6.26 ± 0.02 ($n=6$) and 4.83 ± 0.02 ($n=6$). Among these agonists, 2-methylthio ATP displayed the lowest apparent efficacy.

7 The CRC of 2-methylthio ATP in rat duodenum was shifted to the right by PPADS (10-100 μM) in a concentration-dependent manner, and Schild analysis gave a pA_2-value of 5.09 ± 0.06 (slope = 1.02, $n=14$).

8 PPADS was without any effect on the carbachol-induced contraction in guinea-pig taenia coli or rat duodenum and on the relaxation to noradrenaline or adenosine in guinea-pig taenia coli.

9 In conclusion, the antagonistic properties of PPADS at the taenia coli and rat duodenum P_{2y}-purinoceptors were different from those recently described at the P_{2x}-subtype: inhibition of P_{2y}-purinoceptor-mediated responses was observed at higher concentrations (3-100 μM vs. 1-10 (30) μM). Furthermore, we conclude that in addition to the classical P_{2y}-subtype, which is largely PPADS-resistant, the guinea-pig taenia coli may be endowed with a distinct relaxation-mediating P_2-purinoceptor subtype which is sensitive to PPADS.

Keywords: PPADS; 2-methylthio ATP; ATP; α,β-methylene ATP; carbachol; P_{2y}-purinoceptor; electrical field stimulation; ecto-nucleotidase; guinea-pig taenia coli; rat duodenum

Introduction

Since the pharmacological P_2-purinoceptor subclassification (P_{2x}, P_{2y}), which is based on the rank order of potencies of different agonists (Burnstock & Kennedy, 1985; Abbracchio & Burnstock, 1994), and the recent cloning of several P_2-purinoceptor subtypes (Webb *et al.*, 1993; Lustig *et al.*, 1993; Valera *et al.*, 1994; Brake *et al.*, 1994; Parr *et al.*, 1994; Filtz *et al.*, 1994) there has been a permanent lack of highly selective, competitive P_2-purinoceptor antagonists. Although suramin has been used as an antagonist of P_2-purinoceptor-mediated responses in numerous studies (Dunn & Blakeley, 1988; Den Hertog *et al.*, 1989; Hoyle *et al.*, 1990; Hoyle & Edwards, 1992; Valera *et al.*, 1994), it does not discriminate between the P_{2x}- and the P_{2y}-subtype. α,β-Methylene ATP has been used as a specific desensitizing agent to block P_{2x}-mediated effects (Ralevic & Burnstock, 1988; O'Connor *et al.*, 1990). Reactive blue 2, which antagonizes P_{2y}-mediated responses, can be used only within a narrow concentration-range, above which it displays non-specific effects (Burnstock & Warland, 1987; but see Bültmann & Starke, 1994b). Arylazidoaminopropionyl ATP ($ANAPP_3$), a photoaffinity label, and 4,4'-diisothiocyanato-

[1] Author for correspondence.

stilbene-2,2'-disulphonate (DIDS) irreversibly block the P_{2x}-subtype (Hogaboom *et al.*, 1980; Bültmann & Starke, 1994a). Finally, the ATP analogue, 2-propylthio-D-β,γ-difluoromethylene ATP (FPL 66096), has been reported to be a potent and highly selective P_{2t}-purinoceptor antagonist (Humphries *et al.*, 1994).

Recently, the compound pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) has been described as a novel functionally-selective antagonist of P_2-purinoceptor-mediated responses in rabbit vas deferens (Lambrecht *et al.*, 1992). PPADS postsynaptically antagonizes P_{2x}-mediated responses, generated either by exogenously applied α,β-methylene ATP or by electrical field stimulation. Moreover, PPADS increases noradrenaline overflow via a putative presynaptic P_2-purinoceptor (Grimm *et al.*, 1994). In these studies, no interaction with either α_1-adrenoceptors, muscarinic M_1-(M_4), M_2- and M_3-receptors, histamine H_1- or adenosine A_1-receptors was found. Thus, PPADS seems to be fairly specific for P_2-purinoceptors. Subsequent studies on guinea-pig ileum resistance vessels (Bungardt *et al.*, 1992), guinea-pig vas deferens (McLaren *et al.*, 1993; 1994), rabbit urinary bladder (Ziganshin *et al.*, 1993), various rabbit isolated blood vessels (Ziganshin *et al.*, 1994a) and rat mesenteric arterial bed (Windscheif *et al.*, 1994b, c) have confirmed a specific antagonism for the P_{2x}-purinoceptor. In addition, the last two studies provide evidence for PPADS being more or less ineffective at the vascular P_{2y}-purinoceptor. PPADS was also shown to be devoid of an effect at P_{2u}-purinoceptors in rat mesenteric arterial bed (Windscheif *et al.*, 1994b, c) and rat isolated lung (Rubino & Burnstock, 1994). Furthermore, PPADS inhibits ADP-induced human platelet aggregation at excessive concentrations only (>100 μM; Windscheif *et al.*, 1994a). It is noteworthy, that the synthesis precursor of PPADS, pyridoxal-5-phosphate, and an isomer of PPADS, pyridoxalphosphate-6-azophenyl-2',5'-disulphonic acid (iso-PPADS) act as antagonists of P_{2x}-purinoceptor-mediated responses (Bültmann & Starke, 1994b; Khakh *et al.*, 1994; Trezise *et al.*, 1994a, b). However, little is known about the P_2-purinoceptor-selectivity of these compounds.

The present study was undertaken in order to assess further the selectivity of PPADS for different P_2-purinoceptor subtypes. Thus, the effect of PPADS on the relaxant response to 2-methylthio ATP, ATP, α,β-methylene ATP, adenosine and noradrenaline as well as electrical field stimulation was examined in the carbachol-contracted guinea-pig taenia coli, a tissue that is endowed with the archetypal P_{2y}-purinoceptor (Burnstock & Kennedy, 1985). Moreover, PPADS was investigated for its effect on the relaxant response to 2-methylthio ATP in the rat duodenum, another functional P_{2y}-purinoceptor model (Hourani *et al.*, 1991). Since ecto-nucleotidases, dephosphorylating ATP and 2-methylthio ATP, have been shown to be present in the taenia coli (Welford *et al.*, 1986), a putative inhibitory effect of PPADS on ecto-enzyme activity was also investigated in this tissue.

A preliminary account of these results has been presented elsewhere (Windscheif *et al.*, 1994c; Ziyal *et al.*, 1994).

Methods

Guinea-pig taenia coli

Male Dunkin Hartley guinea-pigs, 300–600 g, were killed by a blow to the head and exsanguination. The taenia was dissected free without opening the caecum. Segments 1.5–2.0 cm long were suspended in 10 ml organ-baths containing modified Krebs solution of the following composition (mM): NaCl 133.5, KCl 4.7, Na_2HPO_4 0.8, $NaHCO_3$ 16.3, $MgSO_4$ 0.6, $CaCl_2$ 2.5 and glucose 7.8. The Krebs solution was gassed with 95% O_2/5% CO_2 and temperature was kept at 36–37°C.

After preloading the taenia strips with 1 g tension they were allowed to equilibrate for 45–60 min. Electrical field stimulation (EFS) was applied via two platinum-wire rings 10 mm apart, through which the tissue was threaded. Mechanical activity was recorded isometrically by a Grass Force Displacement Transducer FT03C and displayed on a Grass 79D polygraph.

A standard tone was induced by carbachol (50 nM, occasionally up to 70 nM to maintain a constant precontraction) every 10 min. When the carbachol contraction reached a plateau either a single dose of relaxant agonist or EFS (80 V, 0.3 ms, at 0.5, 2, 8, or 16 Hz for 5 s) was applied. As soon as a maximum response was observed the organ-baths were washed repeatedly. The first dose of relaxant agonist was a priming dose (causing >50% relaxation); its effect was ignored. In EFS-experiments a train of pulses at 8 Hz was used to prime the tissue. Again, this effect was ignored. Throughout the experiments, doses of agonists and EFS were applied in a randomized manner. Only one relaxant agonist was tested on one preparation; EFS was carried out in parallel with one relaxant agonist on the same strip. After a full concentration- or frequency-response curve had been obtained, PPADS was incubated for 30 min (with washing and readministration of PPADS after 15 min) and a second curve was constructed in the presence of PPADS. With several preparations a second concentration of PPADS was incubated (30 min) and a third CRC was constructed. On at least four different preparations for each agonist and for EFS, respectively, three concentration- or frequency-response curves were constructed on the same tissue with a time interval of 30 min (no incubation of PPADS). These experiments were considered as time-matched controls.

Rat duodenum

Male Wistar rats, 250–300 g, were killed by cervical dislocation. Approximately 1 cm below the pylorus, segments (1.5 cm) of the duodenum were quickly removed, cleared of connective tissue and placed under a resting tension of 1 g in a 5 ml organ bath containing a modified Krebs solution of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.6, KH_2PO_4 1.2, $NaHCO_3$ 25.0, and glucose 11.1. Both ends of a segment were tied off, so that the lumen was closed. The Krebs solution was bubbled with 95% O_2/5% CO_2 and temperature was kept at 35°C.

Tissues were allowed to equilibrate for 30 min before the addition of drugs. Mechanical activity was recorded isotonically with a force-displacement transducer (TFGViso, W. Fleck, Mainz, Germany) connected to a Hellige amplifier and a Rikadenki pen recorder (Hellige, Freiburg, Germany).

A standard tone was induced with carbachol (100 nM) every 15 min. After reaching a plateau, a single dose of relaxant agonist was applied and washed out immediately after the response had developed. The first dose of agonist was a priming dose; its effect was discarded. The concentration-response relationship for 2-methylthio ATP was examined in preparations before and after 30 min incubation with PPADS (10–100 μM). On 12 preparations, a second CRC of 2-methylthio ATP was constructed after 30 min without incubation with PPADS. These experiments were considered as time-matched controls.

Ecto-nucleotidase assay

In this assay (according to Ziganshin *et al.*, 1994b), the degradation of ATP by ecto-nucleotidases was used as a measure of enzyme activity. Briefly, the assay was carried out with taenia coli strips as prepared for the organ-bath experiments at 37°C in HEPES-buffer (pH 7.4) containing (mM): 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 10, NaCl 135, KCl 5, $CaCl_2$ 2, $MgCl_2$ 2 and glucose 10. Pieces of tissue (2–3 mg) were placed in 24-well cell culture dishes in 300 μl buffer and were prewashed for 15–20 min. The prewash buffer was changed for 250 μl buffer containing 0.1 mM ATP, and the tissues were incubated and shaken continuously for 30 min. Incubation was terminated by removing the buffer and adding

it to 0.9 ml of a 2.5% (w/v) solution of sodium dodecyl sulphate (SDS) for inorganic phosphate assay. Tissues were again washed with buffer for 10–15 min and were subsequently incubated for 30 min with 250 μl buffer containing 0.1 mM ATP and a given concentration of PPADS. The buffer was again collected in SDS. For the inorganic phosphate assay, 1 ml of 1.25% (w/v) ammonium molybdate solution in 2 N HCl and 0.1 ml of 16% (w/v) Fiske and SubbaRow reducing agent were added to the samples. The solutions were transferred to cuvettes and were left for 30 min at room temperature to develop colour. The inorganic phosphate produced was measured spectrophotometrically at 700 nm using a Beckman Du-65 spectrophotometer. KH_2PO_4 was used as a phosphate standard. The amount of phosphate produced in the presence of PPADS (second incubation) was calculated as a percentage of that produced in the absence of PPADS (first incubation). Results were obtained from two experiments performed in quadruplicate. Blanks, containing either ATP and no biological material or of the opposite composition, were included in each assay.

When kinetic constants were studied, tissues were incubated initially with a given concentration of ATP (30–3000 μM) for 30 min and then, after collecting samples and washing, were incubated with the same concentration of ATP together with PPADS (100 μM).

Drugs used

Carbamylcholine chloride (carbachol), adenosine 5'-triphosphate sodium salt (ATP), α,β-methylene ATP lithium salt (α,β-meATP), adenosine hemisulphate, (−)-noradrenaline bitartrate, and Fiske and SubbaRow reducing agent were purchased from Sigma Chemical Co., U.K. 2-Methylthio ATP was obtained from Research Biochemicals, Inc., Natick, MA, U.S.A. Pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid (PPADS) was synthesized in one of our laboratories. Noradrenaline was dissolved in 0.1 mM ascorbic acid. The other drugs were dissolved in distilled water.

Analysis of results

The relaxant effect to agonists or EFS was expressed as percentage of the carbachol-induced contraction. Responses to exogenously applied agonists were plotted against agonist concentrations in a semi-logarithmic manner. pD_2-values were calculated as $-\log EC_{50}$. Dose-ratios (DR) were obtained from the individual CRC shifts produced in the presence of PPADS at the level of the respective half-maximum response, and pA_2-values (guinea-pig taenia coli) were calculated according to the equation: $pA_2 = \log (DR-1) + pA_x$ (pA_x being the $-\log$ molar antagonist concentration; Furchgott, 1972). Schild analysis was carried out according to Arunlakshana & Schild (1959). The curves in Figures 2, 5 and 6 were fitted to a logistic function (Parker & Waud, 1971). Ecto-nucleotidase activity was calculated in terms of pmol P_i min^{-1} mg^{-1} wet tissue. The K_m- and V_{max}-values were derived from Lineweaver-Burk plots. Data are presented as means (± s.e.mean where appropriate) from n observations. Means were compared by Student's paired t test. A probability of 0.05 or less was considered significant.

Results

Guinea-pig taenia coli

A standard carbachol concentration (50–70 nM) caused a sustained contraction of the smooth muscle (about 80% of maximum response), which was unaffected by PPADS (100 μM; data not shown).

The adenine nucleotides 2-methylthio ATP, ATP and α,β-meATP caused concentration-dependent relaxation of the carbachol-induced contraction. The calculated pD_2-values were 7.96 ± 0.08 (23), 6.27 ± 0.12 (21) and 5.88 ± 0.04 (24), respectively. Apart from its potency, α,β-meATP could be distinguished from 2-methylthio ATP and ATP with regard to the time course of the response. A rapid and transient response was typical for 2-methylthio ATP and ATP, whereas the response to α,β-meATP developed slowly (Figure 1).

Time-matched controls demonstrated that during the course of the experiments there was no change in tissue sensitivity towards 2-methylthio ATP, ATP and α,β-meATP. The pD_2-values calculated from the first, second and third curve were: 7.76 ± 0.23 (4), 7.87 ± 0.14 (4), 7.78 ± 0.20 (4) for 2-methylthio ATP; 6.68 ± 0.29 (4), 6.62 ± 0.22 (4), 6.62 ± 0.11 (4) for ATP and 5.76 ± 0.09 (5), 5.68 ± 0.09 (5), 5.69 ± 0.07 (5) for α,β-meATP, respectively.

Each of the agonists was tested in the presence of three to four different concentrations of PPADS. PPADS itself had no effect on the resting tone of the preparation, but produced rightward displacements of the CRCs of the agonists without reduction of the maximum response (Figure 2).

For 2-methylthio ATP and ATP, the PPADS concentrations tested were 3, 10, 30 and 100 μM. At 3 μM PPADS, the CRC of 2-methylthio ATP was consistently shifted to the right (Figure 2a) with a mean dose-ratio of 3.6 ± 0.62 and a corresponding pA_2-value of 5.85 ± 0.13 (6). In contrast, there was no consistent rightward shift of the CRC of ATP in the presence of 3 μM PPADS; the individual dose-ratios were 0.56, 0.22, 1.62, and 6.29, respectively. Thus, for ATP there was no significant difference in the mean pD_2-values in the absence or in the presence of 3 μM PPADS (5.36 ± 0.26 (4) vs. 5.34 ± 0.22 (4)). However, 10 and 30 μM PPADS produced shifts of the CRC of 2-methylthio ATP and ATP with moderate and similar dose-ratios (4.1 ± 0.62 and 5.4 ± 0.74 at 10 μM; 4.6 ± 0.70 and 4.0 ± 0.78 at 30 μM; Figure 2a, b). These values corresponded to the respective mean pA_2-values of: 5.44 ± 0.09 (6)/ 5.63 ± 0.08 (4) and 5.02 ± 0.10 (6)/4.94 ± 0.12 (5). PPADS (100 μM) was needed to produce a substantial dose-ratio (25.1 ± 8.37 and 19.3 ± 7.04), the calculated pA_2-values being 5.26 ± 0.16 (5) and 5.15 ± 0.13 (6) against 2-methylthio ATP and ATP, respectively. The biphasic nature of the resulting Schild plots was evident (Figure 3a, b), and the overall slopes derived from Schild analysis were 0.58 ± 0.14 (total data points = 20) and 0.60 ± 0.22 (total data points = 15), respectively, only the former of which was significantly different from unity.

α,β-meATP was tested in the presence of 3, 10 and 30 μM PPADS (Figure 2c). Again, there was little difference between the dose-ratios (2.1 ± 0.20; 3.3 ± 0.55) produced by the two lower concentrations of PPADS. The corresponding pA_2-values were 5.52 ± 0.12 (5) and 5.32 ± 0.10 (5), respectively. PPADS (30 μM), yielded an extensive shift (31.5 ± 6.44) of the CRC corresponding to a pA_2-value of 5.97 ± 0.08 (6). The resulting Schild plot was again biphasic (Figure 3c), although less distinctively. Schild analysis yielded an overall slope of 1.46 ± 0.18 (total data points = 16), which was significantly different from unity.

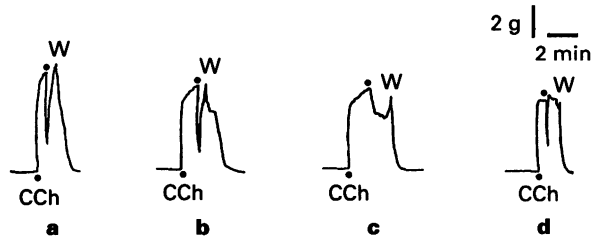

Figure 1 Typical traces demonstrating the time course of the relaxant responses to nucleotide agonists and electrical field stimulation (EFS; 80 V, 0.3 ms, 5 s, 8 Hz) on the carbachol-contracted guinea-pig taenia coli. Dots show the application of carbachol (CCh, 50 nM), 2-methylthio ATP (10 nM; a), ATP (3 μM; b), α,β-methylene ATP (1 μM; c) and EFS (d). W denotes washing.

In order to investigate whether PPADS affected relaxant responses mediated via adrenoceptors or the A_2-purinoceptor (Burnstock *et al.*, 1984), noradrenaline and adenosine were tested in the presence of 100 μM PPADS, the highest concentration used in this study. The pD_2-values in the absence and in the presence of PPADS were not significantly different: 7.22 ± 0.09 (5) and 7.11 ± 0.08 (5) for noradrenaline and 4.36 ± 0.25 (3) and 4.38 ± 0.16 (3) for adenosine, respectively.

In the EFS-experiments, frequency-response curves with a frequency range from 0.5–16 Hz were constructed in the absence and presence of four different concentrations of PPADS (Figure 4). Time-matched controls demonstrated that the response to EFS at either frequency in the first curve was not significantly different from the respective response in the second or third curve. Relaxant responses, rapid and transient (Figure 1), increased from 0.5 Hz ($37.9 \pm 5.4\%$) to 2 Hz ($60.0 \pm 4.3\%$), then reached a plateau at 8 Hz ($64.5 \pm 3.1\%$), and finally started to decline at 16 Hz ($56.0 \pm 3.1\%$, $n = 19-20$; Figure 4). PPADS (3 μM) was without significant effects on EFS-responses at any given frequency. However, 10, 30 and 100 μM PPADS concentration-dependently antagonized EFS-evoked relaxations at the respective frequencies. In the presence of 10 μM PPADS ($n = 9$), these responses were

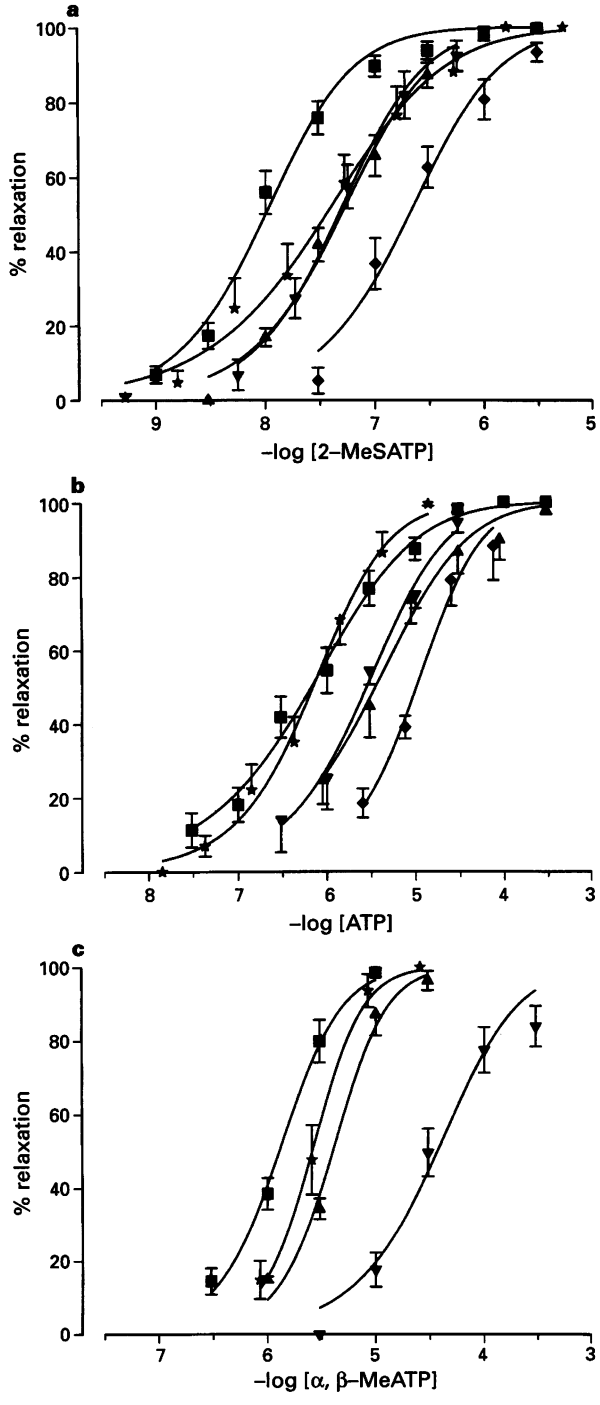

Figure 2 Concentration-response curves of 2-methylthio ATP (2-MeSATP, a), ATP (b) and α,β-methylene ATP (α,β-meATP, c) in the guinea-pig taenia coli in the absence (■) and in the presence of 3 μM (★), 10 μM (▲), 30 μM (▼) and 100 μM (◆) PPADS. Responses are expressed as % relaxation of carbachol contraction. Data shown are mean ± s.e.mean from 4–18 experiments. Some error bars fall within the area covered by a symbol.

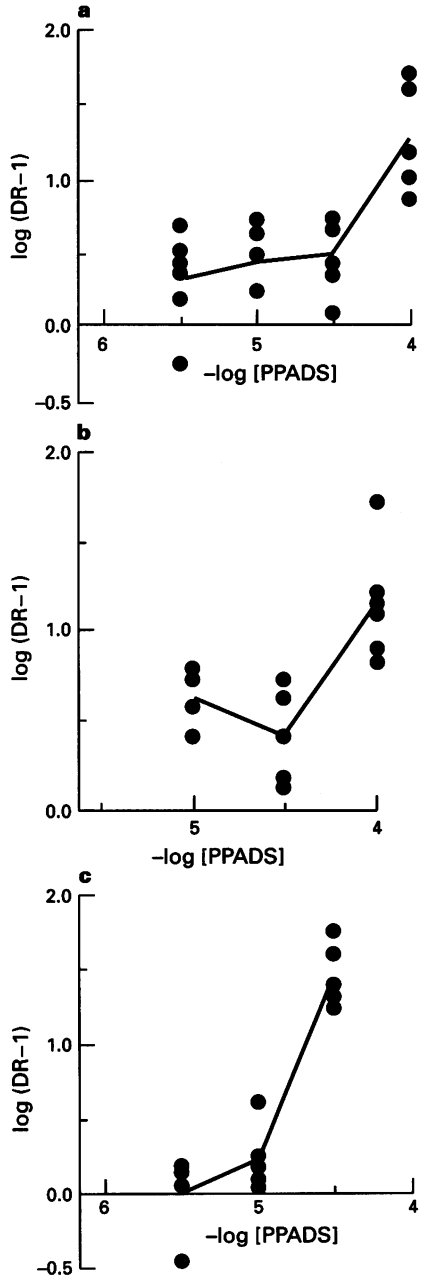

Figure 3 Schild plots for the antagonism by PPADS in the carbachol-contracted guinea-pig taenia coli using three different agonists: 2-methylthio ATP (a), ATP (b) and α,β-methylene ATP (c). Lines, which connect the mean log (DR-1) values at the respective antagonist concentrations, are drawn to highlight the biphasic nature of the Schild plots. The slopes of the overall regression lines (not shown) were: 0.58 ± 0.14 (a); 0.60 ± 0.22 (b); 1.46 ± 0.18 (c).

20.9±3.5% (0.5 Hz), 47.4±7.6% (2 Hz), 52.4±7.0% (8 Hz) and 44.5±7.9% (16 Hz), in the presence of 30 μM PPADS ($n=6$) 11.3±5.6% (0.5 Hz), 14.4±5.8% (2 Hz), 17.8±4.5% (8 Hz) and 23.6±8.3% (16 Hz), and in the presence of 100 μM PPADS ($n=10$) 10.2±4.0% (0.5 Hz), 5.6±3.2% (2 Hz), 4.8±2.5% (8 Hz) and 4.1±2.8% (16 Hz). It should be pointed out, that occasionally at 30 μM and 100 μM PPADS (more frequently) EFS-induced relaxations were converted to transient contractions especially at higher frequencies.

Rat duodenum

In preparations, showing a moderate spontaneous activity, 100 nM carbachol induced a sustained contraction of 50% of its maximum effect, which was unaffected by PPADS (100 μM; data not shown).

2-Methylthio ATP, ATP and α,β-meATP all relaxed the carbachol-contracted rat duodenum, the pD_2-values being 6.98±0.04 (76), 6.26±0.02 (6) and 4.83±0.02 (6), respectively. The maximal reduction of the carbachol-contracted rat duodenum caused by the nucleotides was 64±1.65% (76) for 2-methylthio ATP, 105±1.17% (6) for ATP and 132±2.73% (6) for α,β-meATP (Figure 5).

PPADS (10, 30 and 100 μM) was without effect on the resting tone of the preparation, but produced concentration-related dextral shifts of the CRCs of 2-methylthio ATP (Figure 6). The mean dose-ratios were 2.5±0.41 (5), 5.1±0.86 (5) and 14.3±2.23 (4), respectively. At 100 μM PPADS, the shift was accompanied by an increase in the maximum response to 2-methylthio ATP (97±1.78%, $n=4$ vs. 64±1.65%, $n=76$ in the absence of PPADS). The resulting Schild plot was linear yielding a pA_2-value of 5.09±0.06 (14) and a slope of 1.02±0.16, which was not significantly different from unity (Figure 7).

In time-matched control experiments, the pD_2-values of 2-methylthio ATP calculated from the first and second CRC were not significantly different: 6.82±0.02 (12) and 6.70±0.03 (12), respectively.

Ecto-nucleotidase assay

The spontaneous ATP breakdown when incubated in buffer without tissue as well as the amount of P_i released from the

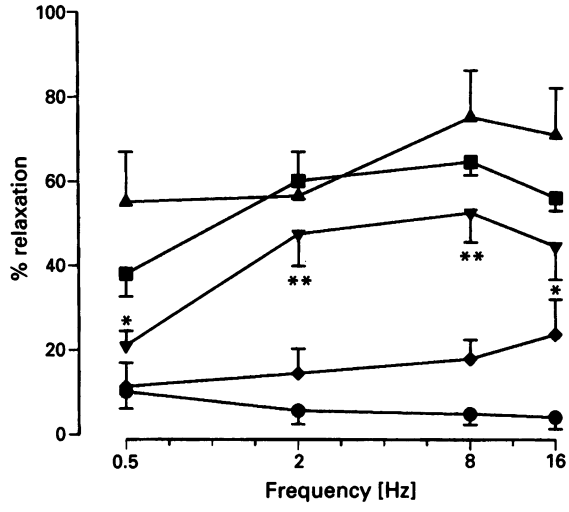

Figure 4 Frequency-response curves (80 V, 0.3 ms, 5 s, 0.5–16 Hz) in the absence (■; $n=19-20$) and in the presence ($n=4-10$) of different concentrations of PPADS (3 μM▲, 10 μM▼, 30 μM◆, 100 μM●) in the guinea-pig taenia coli. Responses are expressed as % relaxation of carbachol contraction. $^*P<0.05$, $^{**}P<0.01$. Note that the x-axis is on a logarithmic scale.

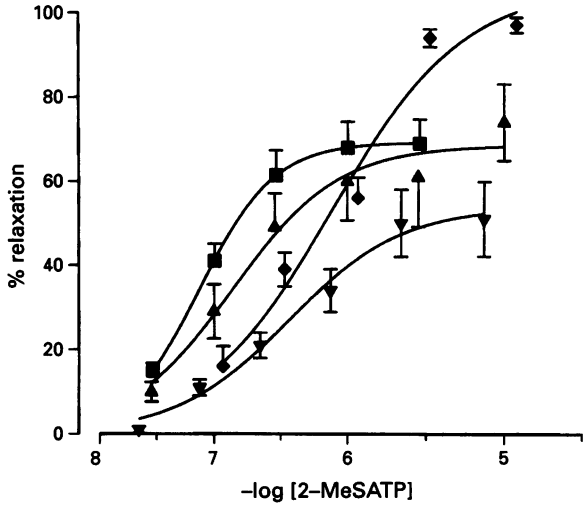

Figure 6 Relaxation of the carbachol-contracted rat duodenum induced by 2-methylthio ATP (2-MeSATP) in the absence (■) and in the presence of PPADS (10 μM ▲, 30 μM ▼, 100 μM ◆). Data shown are mean±s.e.mean ($n=4-5$).

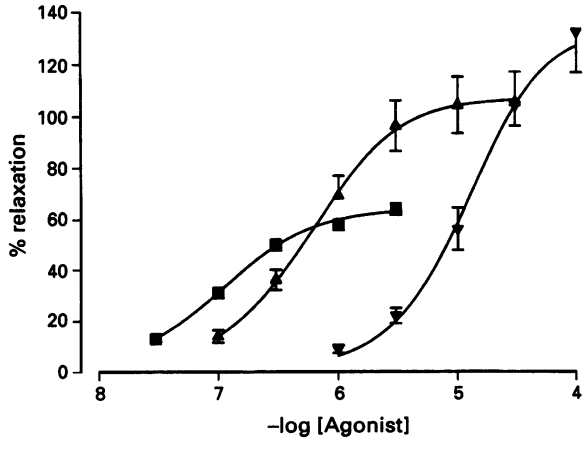

Figure 5 The relaxant effect of 2-methylthio ATP (■), ATP (▲) and α,β-methylene ATP (▼) on the carbachol-contracted rat duodenum. Responses are expressed as % relaxation of carbachol contraction. Data shown are mean±s.e.mean ($n=6-76$). Some error bars fall within the symbols.

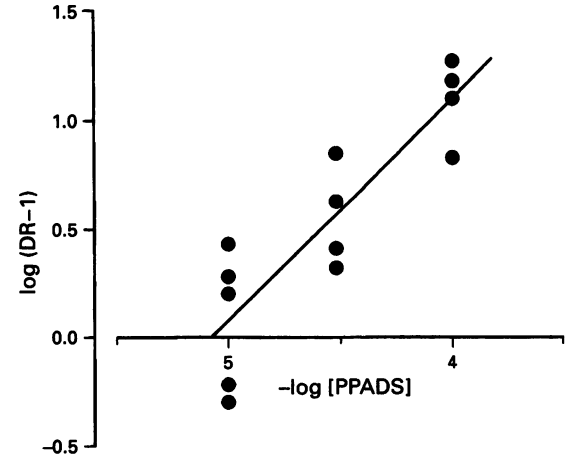

Figure 7 Schild plot for PPADS against 2-methylthio ATP in the carbachol-contracted rat duodenum, yielding a pA_2-value of 5.09±0.06 ($n=14$) and a slope of 1.02±0.16 ($r=0.99$).

tissue during incubation time was negligible (data not shown).

PPADS (3 μM) did not significantly affect ecto-nucleotidase activity in guinea-pig taenia coli; however, at concentrations of 10 μM and above it significantly inhibited enzyme activity. This inhibition was not concentration-dependent, since in the presence of 10, 30 and 100 μM PPADS the ecto-nucleotidase activities were not significantly different from one another (residual enzyme activity: 84.0 ± 3.0% (8), 78.6 ± 5.4% (8) and 81.8 ± 5.1% (8), respectively; Figure 8).

When different concentrations of ATP (30–3000 μM; $n=8$) were used, the ecto-nucleotidases appeared to have a K_m-value of 366 μM and a V_{max}-value of 2.61 nmol P_i min^{-1} mg^{-1}. In the presence of PPADS (100 μM), these constants became 468 μM and 2.02 nmol P_i min^{-1} mg^{-1}, respectively, indicating that this inhibition was not competitive (Figure 9).

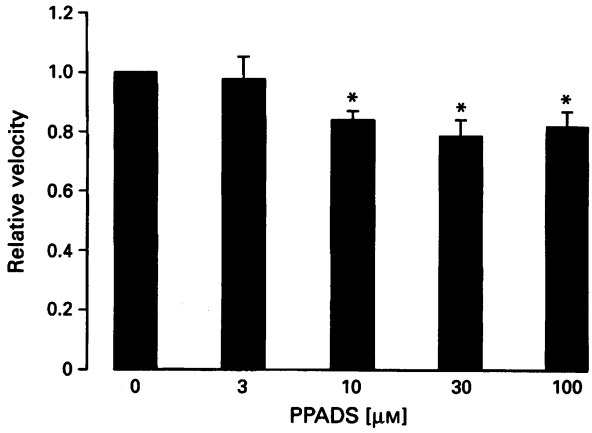

Figure 8 Relative velocities of ecto-nucleotidase activity in guinea-pig taenia coli using ATP (0.1 mM) as substrate in the absence and presence of different concentrations of PPADS. Values shown are means ± s.e.mean of two experiments performed in quadruplicate ($n=8$). The absolute velocity in controls was 385 ± 14 ($n=24$) pmol P_i min^{-1} mg^{-1} wet tissue. A significant difference from control is denoted by * ($P<0.05$).

Discussion

Guinea-pig taenia coli

The present rank order of potency of the adenine nucleotides (2-methylthio ATP > > ATP > α,β-meATP) is compatible with the classical P_{2y}-purinoceptor as defined by Burnstock & Kennedy (1985).

Our results show that PPADS affected the relaxant responses to 2-methylthio ATP and ATP in a similar way, giving biphasic Schild plots with similar pA_2-values at any PPADS concentration. Moreover, PPADS seems to differentiate between the effects induced by the agonists 2-methylthio ATP and ATP on the one hand and α,β-meATP on the other hand. As a result, PPADS was more potent in inhibiting relaxations to α,β-meATP than to 2-methylthio ATP or ATP. In order to compare quantitatively the antagonistic effects of PPADS against the three agonists, it is most reasonable to consider the pA_2-values calculated from similar and substantial shifts of the CRC rather than from dose-ratios at lower PPADS concentrations where these values do not appear to be concentration-related. Accordingly, the pA_2-values of 5.26, 5.15 and 5.97 referring to dose-ratios of 25.1, 19.3, and 31.5 (at the respective highest PPADS concentration) against 2-methylthio ATP, ATP, and α,β-meATP, respectively, were compared. The results show that PPADS was about 6 fold more potent against α,β-meATP than against the other two agonists.

One explanation for the complex nature of antagonism by PPADS could be the ability of PPADS to inhibit breakdown of the nucleotides by ecto-nucleotidases (Welford *et al.*, 1986). Degradation of the nucleotides would eventually lead to the production of metabolites which are active as agonists in their own right, e.g. adenosine, causing relaxation via A_2-receptors (Burnstock *et al.*, 1984). Since A_2-receptor-mediated effects are not inhibited by PPADS (this study), this could easily explain the roughly concentration-independent shifts of the CRCs to either agonist at lower PPADS concentrations. At high nucleotide concentrations (i.e. at high PPADS concentrations), the enzymes might have been saturated, and a substantial shift of the CRCs of the agonists was observed. This explanation would also match the less distinctive biphasic Schild plot using the more stable analogue, α,β-meATP, as the agonist. However, it was unlikely that dephosphorylation of the agonists was functionally implicated during the development of responses, since Hourani *et al.* (1991) were able to show in guinea-pig taenia coli that the nonselective P_1-antagonist 8-(*p*-sulphophenyl) theophylline (8-SPT) did not inhibit responses to any of the agonists used in the present study. In addition, Welford *et al.* (1986) showed that the potency of ATP and a series of ATP analogues is independent of their resistance to hydrolysis by ecto-nucleotidases in guinea-pig taenia coli. However, we examined the ability of PPADS to affect ecto-nucleotidases in the taenia coli. Since the K_m-value (366 μM) was found to be much higher than the EC_{50} of ATP (0.45 μM) for relaxation, it is unlikely that this level of enzyme activity affects the CRC of ATP. At PPADS concentration of 10 μM or

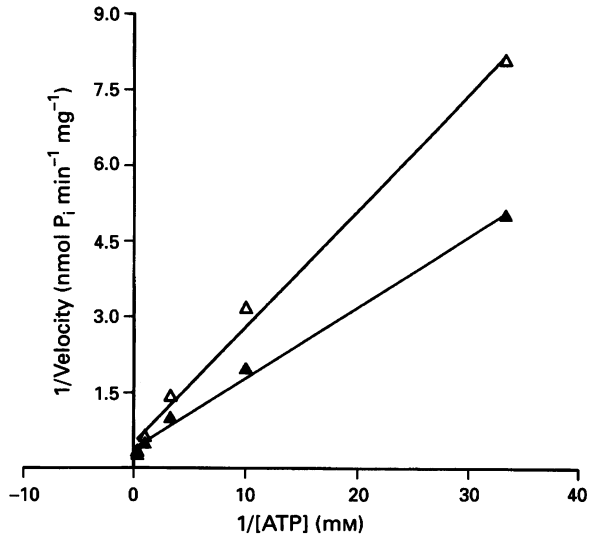

Figure 9 The Lineweaver-Burk plot for guinea-pig taenia coli ecto-nucleotidase, constructed in the absence (▲) and presence of PPADS (100 μM; △). Values shown are means ± s.e.mean of two experiments performed in quadruplicate ($n=8$).

higher, there was indeed some enzyme inhibition. However, this effect was not concentration-related and did not exceed 20%. Collectively, the presence of ecto-nucleotidase activity does not explain the complex nature of functional antagonism by PPADS in guinea-pig taenia coli and the weak inhibition by PPADS of enzyme activity suggests that effects on ecto-nucleotidases will not be a complicating factor if PPADS is used as a P_2-purinoceptor antagonist.

Taking into consideration that the taenia coli relaxation-mediating P_2-purinoceptor(s) behaved differently from the classical P_{2y}-subtype in other whole tissue preparations, e.g. in blood vessels, where PPADS has little or no effect (Ziganshin *et al.*, 1994a; Windscheif *et al.*, 1994b, c) and considering the recent postulation of subtypes of the P_{2y}-purinoceptor (Abbracchio & Burnstock, 1994; Boyer *et al.*, 1994), we hypothesize that in addition to the classical P_{2y}-subtype, which is largely PPADS-resistant, the guinea-pig taenia coli is endowed with a distinct relaxation-mediating, PPADS-sensitive P_2-purinoceptor subtype. This hypothesis is based on the following findings. (1) A biphasic nature of each Schild plot was evident, strongly suggesting receptor heterogeneity. (2) PPADS ob-

viously differentiates between the effects induced by the three agonists used (see above). (3) The differences in the time course of the responses (α,β-meATP revealed a distinctively slower onset) provide indication towards the possibility of two mechanisms of action amongst the three agonists.

Interestingly, there has been an early report using an electrophysiological approach and claiming two different mechanisms of α,β-meATP action in guinea-pig taenia coli, one of which is shared by other nucleotides (Den Hertog *et al.*, 1985). Most recently, it was reported that ATP and 2-methylthio ATP – in contrast to α,β-meATP – may produce relaxation of the taenia coli via suramin-sensitive and -insensitive mechanisms (Kelley & Hollingsworth, 1994). It is also noteworthy, that Hourani *et al.* (1991) found a pronounced difference in β,γ-meATP activity in guinea-pig taenia coli and rat duodenum, respectively. Whereas in the presence of 8-SPT (100 μM), relaxations to β,γ-meATP were only slightly antagonized in the taenia, they were abolished in duodenum. The authors raise the question whether this reflects a 'qualitative difference between the P_{2y}-receptor populations in these tissues'. However, they do not see a 'compelling reason' to propose different subtypes with the pharmacological tools available. In addition, Dudeck *et al.* (1995) reported that there are at least two relaxation-mediating P_2-purinoceptors in guinea-pig taenia coli, the P_{2y}-receptor being insensitive to DIDS and a distinct receptor for α,β-meATP being very sensitive to DIDS. All these studies support our conclusion of receptor heterogeneity (Windscheif *et al.*, 1994c and this study).

In the present study, PPADS caused a rightward shift of the CRC of the nucleotides without depression of the maximum effect. This contrasts with PPADS-antagonism at P_{2x}-purinoceptors in rabbit vas deferens (Lambrecht *et al.*, 1992) and guinea-pig ileum resistance vessels (Bungardt *et al.*, 1992) which is insurmountable. In rabbit urinary bladder (Ziganshin *et al.*, 1993), various rabbit isolated blood vessels (Ziganshin *et al.*, 1994a) and rat mesenteric arterial bed (Windscheif *et al.*, 1994b,c) the maximum response to α,β-meATP was not reached in the presence of PPADS (3–30 μM). However, in the guinea-pig vas deferens (McLaren *et al.*, 1993; 1994) PPADS (30 μM) did not reduce the maximum response to exogenous α,β-meATP. This is in line with the present study. In contrast to the antagonistic activity of PPADS at P_{2x}-purinoceptors, higher concentrations (3–100 μM vs. 1–10(3) μM) were necessary to block the classical P_{2y}-subtype in the taenia coli. However, a true affinity estimate for the P_{2y}-subtype in this tissue, could not be obtained because of the complex nature of antagonism.

The responses to EFS were affected by PPADS at the same concentration-range as were the responses to exogenous 2-methylthio ATP and ATP. This supports the purinergic nerve hypothesis, which postulates ATP or a related compound as NANC-transmitter (Burnstock *et al.*, 1970; for recent reviews see Hoyle & Burnstock, 1991 and Hoyle, 1992). Also, nitric oxide (NO) has been discussed as NANC-transmitter in the gut. However, in the guinea-pig taenia coli the predominant part of the inhibitory NANC response is not mediated by NO (Knudsen & Tøttrup, 1992).

The observation that relaxations to EFS occasionally were converted to transient contractions, especially at higher frequencies and higher PPADS concentrations, could be explained by revealing the effect of cholinergic excitation while blocking NANC relaxation by PPADS. Acetylcholine is released as an excitatory transmitter by EFS causing contraction of the taenia coli (Knudsen & Tøttrup, 1992). This effect could not be abolished by atropine in the present study because the experiments were carried out on the carbachol-contracted taenia coli. In this regard, histamine might prove to be a useful agent to induce a standard tone in the preparation in the presence of atropine. Further experiments are needed to clarify this issue.

In addition, PPADS was without any influence on adrenoceptor- or A_2-purinoceptor-mediated relaxations. The respective pD_2-values for noradrenaline and adenosine in the absence and presence of 100 μM PPADS, the highest concentration used in this study, were not significantly different. Furthermore, carbachol-contraction remained unaffected, thus, also excluding an interaction with postsynaptically located muscarinic M_3-receptors (Elnatan & Mitchelson, 1993).

Rat duodenum

The rank order of potency of the adenine nucleotides (2-methylthio ATP > ATP > > α,β-meATP) confirmed the suggestion that the receptor involved in relaxation of the rat duodenum is of the P_{2y}-purinoceptor subtype (Hourani *et al.*, 1991; Johnson & Hourani, 1994). The agonists showed different apparent efficacies with 2-methylthio ATP being the most potent but the least efficacious, which is in agreement with a study by Hourani *et al.* (1991). This property of 2-methylthio ATP, i.e. low apparent efficacy but high potency, has been observed in a number of tissues, such as precontracted bovine aortic collateral artery rings (Wilkinson *et al.*, 1994) and precontracted rat aortic rings (O'Connor *et al.*, 1991). In these tissues, a 'mixed' receptor population mediating the same functional response was claimed, i.e. 2-methylthio ATP being active as P_{2y}- and inactive at nucleotide receptors. However, the rat duodenum is unlikely to possess a relaxation-mediating nucleotide receptor because UTP is much less potent than ATP (Johnson & Hourani, 1994).

PPADS (10–100 μM) reversibly blocked 2-methylthio ATP-induced relaxations in rat duodenum. The slope of 1.02 of the respective Arunlakshana-Schild regression argues for a competitive antagonism. The calculated pA_2-value of 5.09 for PPADS is clearly lower compared to the apparent pK_B-values reported for the P_{2x}-subtype (5.62, McLaren *et al.*, 1994; 6.34, Lambrecht *et al.*, 1992), thus, supporting the functional selectivity for PPADS at the P_{2x}-subtype (see also : Windscheif *et al.*, 1994b; Ziganshin *et al.*, 1994a).

At a concentration of 100 μM, PPADS had a second effect: relaxant responses to 2-methylthio ATP were markedly increased. Similar observations have been made for suramin (10–100 μM; $pA_2 = 5.02$) in rat duodenum (Ziyal *et al.*, 1994). Inhibition by PPADS (100 μM) of 2-methylthio ATP degradation by ecto-nucleotidases might explain this phenomenon. However, as in the taenia, a functional implication of nucleotide breakdown in rat duodenum was excluded (Hourani *et al.*, 1991). In addition, PPADS was shown to exhibit only a limited ecto-nucleotidase inhibiting effect (this study) which makes this explanation unlikely. Alternatively, the increase by PPADS (100 μM) of the maximum response to 2-methylthio ATP may be due to the presence of a heterogeneous P_2-purinoceptor population in the rat duodenum: a relaxation-mediating P_{2y}- and a contraction-mediating P_2-purinoceptor both being activated by 2-methylthio ATP, but with PPADS being able to interfere with the latter only at high concentrations. In this case, however, a non-linear Schild plot would have been expected. In addition, no contractions were observed with 2-methylthio ATP (up to 300 μM) in rat duodenum (Johnson & Hourani, 1994). Taken together, the present results are not compatible with the hypotheses that inhibition by PPADS of ecto-nucleotidases in rat duodenum or blockade by PPADS (100 μM) of 2-methylthio ATP-sensitive contraction-mediating P_2-purinoceptors could account for the increase in maximum response to the agonist. Further experiments are needed to clarify this issue.

Even the highest concentration of PPADS (100 μM) was without influence on the carbachol-induced contraction, showing that PPADS is devoid of any effect on functional muscarinic M_3-receptors in rat duodenum (Gross *et al.*, 1994).

In conclusion, the present study describes PPADS as a highly specific P_2-purinoceptor antagonist. This is consistent with previous observations (Lambrecht *et al.*, 1992; Bungardt *et al.*, 1992; McLaren *et al.*, 1993; 1994; Ziganshin *et al.*, 1993; 1994a; Grimm *et al.*, 1994; Windscheif *et al.*, 1994b, c) which exclude any effect of PPADS on α_1-adrenoceptors, muscarinic M_1-(M_4-), M_2- and M_3-receptors, histamine H_1- or adenosine

A_1-receptors. Additionally, in this study PPADS was shown to be devoid of any effect on adenosine A_2-receptors and to exhibit only a weak ecto-nucleotidase inhibitory activity. The present results also confirm that PPADS is able to discriminate between P_{2x}- and the classical P_{2y}-purinoceptor subtypes (this study and the above cited references). Furthermore, this is a study which lends support by means of an antagonist that there are at least two relaxation-mediating P_2-purinoceptors in guinea-pig taenia coli, the classical P_{2y}-purinoceptor which is largely PPADS-resistant and a distinct, PPADS-sensitive P_2-purinoceptor subtype. Several observations argue for the view that the latter does not belong to the family of P_{2x}-purinoceptors (Dudeck *et al.*, 1995). All these results imply that PPADS represents a useful tool for investigating P_2-purinoceptor heterogeneity, and that the guinea-pig taenia coli and rat duodenum should no longer be regarded as functionally equivalent P_{2y}-purinoceptor models.

The authors thank the Fonds der Chemischen Industrie (Germany) (G. L., E. M.), the Deutsche Forschungsgemeinschaft (H. G. B., G.L., O.P.), and the Wellcome Trust (A.U.Z.) for financial support.

References

ABBRACCHIO, M.P. & BURNSTOCK, G. (1994). Purinoceptors: are there families of P_{2x} and P_{2y} purinoceptors? *Pharmacol. Ther.*, **64**, 445–475.

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.*, **14**, 48–58.

BOYER, J.L., ZOHN, I.E., JACOBSON, K.A. & HARDEN, T.K. (1994). Differential effects of P_2-purinoceptor antagonists on phospholipase C- and adenylyl cyclase-coupled P_{2y}-purinoceptors. *Br. J. Pharmacol.*, **113**, 614–620.

BRAKE, A.J., WAGENBACH, M.J. & JULIUS, D. (1994). New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature*, **371**, 519–523.

BÜLTMANN, R. & STARKE, K. (1994a). Blockade by 4,4′-diisothiocyanatostilbene-2,2′-disulphonate (DIDS) of P_{2x}-purinoceptors in rat vas deferens. *Br. J. Pharmacol.*, **112**, 690–694.

BÜLTMANN, R. & STARKE, K. (1994b). P_2-Purinoceptor antagonists discriminate three contraction-mediating receptors for ATP in rat vas deferens. *Naunyn-Schmied. Arch. Pharmacol.*, **349**, 74–80.

BUNGARDT, E., MOSER, U., SPATZ-KÜMBEL, G., BÄUMERT, H.G., LAMBRECHT, G. & MUTSCHLER, E. (1992). Characterization of purinoceptors in guinea-pig ileum resistance vessels by the use of computer-assisted videomicroscopy. *Int. J. Purine Pyrimidine Res.*, **3**, 66.

BURNSTOCK, G., CAMPBELL, G., SATCHELL, D. & SMYTHE, A. (1970). Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br. J. Pharmacol.*, **40**, 668–688.

BURNSTOCK, G., HILLS, J.M. & HOYLE, C.H.V. (1984). Evidence that the P_1-purinoceptor in the guinea-pig taenia coli is an A_2-subtype. *Br. J. Pharmacol.*, **81**, 533–541.

BURNSTOCK, G. & KENNEDY, C. (1985). Is there a basis for distinguishing two types of P_2-purinoceptor? *Gen. Pharmacol.*, **16**, 433–440.

BURNSTOCK, G. & WARLAND, J.J.I. (1987). P_2-purinoceptors of two subtypes in the rabbit mesenteric artery: reactive blue 2 selectively inhibits responses mediated via the P_{2y}- but not the P_{2x}-purinoceptor. *Br. J. Pharmacol.*, **90**, 383–391.

DEN HERTOG, A., NELEMANS, A. & VAN DEN AKKER, J. (1989). The inhibitory action of suramin on the P_2-purinoceptor response in smooth muscle cells of guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **166**, 531–534.

DEN HERTOG, A., PIELKENROOD, J. & VAN DEN AKKER, J. (1985). Effector mechanisms for α,β-methylene ATP and ATP derivatives in guinea-pig taenia caeci. *Eur. J. Pharmacol.*, **110**, 95–101.

DUDECK, O., BÜLTMANN, R. & STARKE, K. (1995). Two relaxation-mediating P_2-purinoceptors in guinea-pig taenia caeci. *Naunyn-Schmied. Arch. Pharmacol.*, **351**, 107–110.

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.

ELNATAN, A. & MITCHELSON, F. (1993). The interaction of McN-A-343 with muscarine receptors in cardiac and smooth muscle. *Biochem. Pharmacol.*, **46**, 933–1003.

FILTZ, T.M., LI, Q., BOYER, J.L., NICHOLAS, R.A. & HARDEN, T.K. (1994). Expression of a cloned P_{2y} purinergic receptor that couples to phospholipase C. *Mol. Pharmacol.*, **46**, 8–14.

FURCHGOTT, R.F. (1972). The classification of adrenoceptors (adrenergic receptors). An evaluation from the standpoint of receptor theory. In *Catecholamines. Handb. Exp. Pharmacol.*, ed. Blaschko, H.G. & Muscholl, E., Vol. 33. pp. 283–335. New York: Springer-Verlag.

GRIMM, U., FUDER, H., MOSER, U., BÄUMERT, H.G., MUTSCHLER, E. & LAMBRECHT. G. (1994). Characterization of the prejunctional muscarinic receptors mediating inhibition of evoked release of endogenous noradrenaline in rabbit isolated vas deferens. *Naunyn-Schmied. Arch. Pharmacol.*, **349**, 1–10.

GROSS, J., PFAFF, O., WAELBROECK, M., CAMUS, J., TASTENOY, M., TACKE, R., MUTSCHLER, E. & LAMBRECHT, G. (1994). Characterization of muscarinic receptors in rat duodenum. *Naunyn-Schmied. Arch. Pharmacol.*, **349** (Suppl.), R75.

HOGABOOM, G.K., O'DONNELL, J.P. & FEDAN, J.S. (1980). Purinergic receptors: Photoaffinity analog of adenosine triphosphate is a specific adenosine triphosphate antagonist. *Science*, **208**, 1273–1276.

HOURANI, S.M.O., BAILEY, S.J., NICHOLLS, J. & KITCHEN, I. (1991). Direct effects of adenylyl 5′-(β,γ-methylene) diphosphonate, a stable ATP analogue on relaxant P_1-purinoceptors in smooth muscle. *Br. J. Pharmacol.*, **104**, 685–690.

HOYLE, C.H.V. (1992). Transmission: purines. In *The Autonomic Nervous System*, Vol. 1: *Autonomic Neuroeffector Mechanisms*. ed. Burnstock, G. & Hoyle, C.H.V. pp. 367–407. London: Harwood Academic Publishers.

HOYLE, C.H.V. & BURNSTOCK, G. (1991). ATP receptors and their physiological roles. In *Adenosine in the Nervous System*. ed. Stone, T.W. pp. 43–76. London: Academic Press Ltd.

HOYLE, C.H.V. & EDWARDS, G.A. (1992). Activation of P_1- and P_{2y}-purinoceptors by ADP-ribose in the guinea-pig taenia coli, but not of P_{2x}-purinoceptors in the vas deferens. *Br. J. Pharmacol.*, **107**, 367–374.

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.

HUMPHRIES, R.G., TOMLINSON, W., INGALL, A.H., CAGE, P.A. & LEFF, P. (1994). FPL 66096: a novel, highly potent and selective antagonist at human platelet P_{2T}-purinoceptors. *Br. J. Pharmacol.*, **113**, 1057–1063.

JOHNSON, C.R. & HOURANI, S.M.O. (1994). Contractile effects of uridine 5′-triphosphate in the rat duodenum. *Br. J. Pharmacol.*, **113**, 1191–1196.

KHAHK, B.S., MICHEL, A. & HUMPHREY, P.A. (1994). Estimates of antagonist affinities at P_{2x} purinoceptors in rat vas deferens. *Eur. J. Pharmacol.*, **263**, 301–309.

KELLY, A.S. & HOLLINGSWORTH, M. (1994). Suramin antagonises ATP and ATP analogues at the P_{2y}-purinoceptors of the guinea-pig taenia caeci. *Can. J. Physiol. Pharmacol.*, **72**, (Suppl. 1), 504.

KNUDSEN, M.A. & TØTTRUP, A. (1992). A possible role of the L-arginine-nitric oxide pathway in the modulation of cholinergic transmission in the guinea-pig taenia coli. *Br. J. Pharmacol.*, **107**, 837–841.

LAMBRECHT, G., FRIEBE, T.H., GRIMM, U., WINDSCHEIF, U., BUNGARDT, E., HILDEBRANDT, C., BÄUMERT, H.G., SPATZ-KÜMBEL, G. & MUTSCHLER, E. (1992). PPADS, a novel functionally selective antagonist of P_2 purinoceptor-mediated responses. *Eur. J. Pharmacol.*, **217**, 217–219.

LUSTIG, K.D., SHIAU, A.K., BRAKE, A.J. & JULIUS, D. (1993). Expression cloning of an ATP receptor from mouse neuroblastoma cells. *Proc. Natl. Acad. Sci. U.S.A.*, **90**, 5113–5117.

MCLAREN, G.J., LAMBRECHT, G., MUTSCHLER, E., BÄUMERT, H.G., SNEDDON, P. & KENNEDY, C. (1994). Investigation of the actions of PPADS, a novel P_{2x}-purinoceptor antagonist, in the guinea-pig isolated vas deferens. *Br. J. Pharmacol.*, **111**, 913–917.

MCLAREN, G.J., SNEDDON, P., KENNEDY, C., LAMBRECHT, G., BURNSTOCK, G., MUTSCHLER, E., BÄUMERT, H.G. & HOYLE, C.H.V. (1993). Investigation of a putative P_{2x}-purinoceptor antagonist in the guinea-pig vas deferens. *Br. J. Pharmacol.*, **109**, 109P.

O'CONNOR, S.E., DAINTY, I.A. & LEFF, P. (1991). Further subclassification of ATP receptors based on agonist studies. *Trends Pharmacol. Sci.*, **12**, 137–141.

O'CONNOR, S.E., WOOD, B.E. & LEFF, P. (1990). Characterization of P_{2x}-receptors in rabbit isolated ear artery. *Br. J. Pharmacol.*, **101**, 640–644.

PARKER, R.B. & WAUD, D.R. (1971). Pharmacological estimation of drug-receptor dissociation constants. Statistical evaluation. I. Agonists. *J. Pharmacol. Exp. Ther.*, **177**, 1–12.

PARR, C.E., SULLIVAN, D.M., PARADISO, A.M., LAZAROWSKI, E.R., BURCH, L.H., OLSEN, J.C., ERB, L., WEISMAN, G.A., BOUCHER, R.C. & TURNER, J.T. (1994). Cloning and expression of a human P_{2u} nucleotide receptor, a target for cystic fibrosis pharmacotherapy. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 3275–3279.

RALEVIC, V. & BURNSTOCK, G. (1988). Actions mediated by P_2-purinoceptor subtypes in the isolated perfused mesenteric bed of the rat. *Br. J. Pharmacol.*, **95**, 637–645.

RUBINO, A. & BURNSTOCK, G. (1994). A new purinergic receptor may contribute to the control of pulmonary circulation of the rat. *Drug Dev. Res.*, **31**, 315.

TREZISE, D.J., KENNEDY, I. & HUMPHREY, P.P.A. (1994a). The use of antagonists to characterize the receptors mediating depolarization of the rat isolated vagus nerve by α,β-methyleneadenosine 5'-triphosphate. *Br. J. Pharmacol.*, **112**, 282–288.

TREZISE, D.J., BELL, N.J., KHAKH, B.S., MICHEL. A.D. & HUMPHREY, P.P.A. (1994b). P_2 purinoceptor antagonist properties of pyridoxal-5-phosphate. *Eur. J. Pharmacol.*, **259**, 295–300.

VALERA, S., HUSSY, N., EVANS, R.J., ADAMI, N., NORTH, R.A., SURPRENANT, A. & BUELL, G. (1994). A new class of ligand-gated ion channel defined by P_{2x} receptor for extracellular ATP. *Nature*, **371**, 516–519.

WEBB, T.E., SIMON, J., KRISHEK, B.J., BATESON, A.N., SMART, T.G., KING, B.F., BURNSTOCK, G. & BANARD, E.A. (1993). Cloning and functional expression of a brain G-protein-coupled ATP receptor. *FEBS Lett.*, **324**, 219–225.

WELFORD, L.A., CUSACK, N.J. & HOURANI, S.M.O. (1986). ATP analogues and the guinea-pig taenia coli: a comparison of the structure-activity relationships of ectonucleotidases with those of P_2-purinoceptor. *Eur. J. Pharmacol.*, **129**, 217–224.

WILKINSON, G.F., MCKECHNIE, K., DAINTY, I.A. & BOARDER, M.R. (1994). P_{2y} purinoceptor and nucleotide receptor-induced relaxation of precontracted bovine aortic collateral artery rings: differential sensitivity to suramin and indomethacin. *J. Pharmacol. Exp. Ther.*, **268**, 881–887.

WINDSCHEIF, U., RADZIWON, P., BREDDIN, H.K., BÄUMERT, H.G., LAMBRECHT, G. & MUTSCHLER, E. (1994a). Two different inhibitory effects of pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid on ADP-induced human platelet aggregation. *Arzneim.-Forsch. Drug Res.*, (in press).

WINDSCHEIF, U., RALEVIC, V., BÄUMERT, H.G., MUTSCHLER, E., LAMBRECHT, G. & BURNSTOCK, G. (1994b). Vasoconstrictor and vasodilator responses to various agonists in the rat perfused mesenteric arterial bed: selective inhibition by PPADS of contractions mediated via P_{2x}-purinoceptors. *Br. J. Pharmacol.*, **113**, 1015–1021.

WINDSCHEIF, U., RALEVIC, V., HOYLE, C.H.V., BÄUMERT, H.G., MUTSCHLER, E., LAMBRECHT, G. & BURNSTOCK, G. (1994c). Functional studies on the P_2-purinoceptor subtype selectivity of pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid. *Drug Dev. Res.*, **31**, 334.

ZIGANSHIN, A.U., HOYLE, C.H.V., BO, X., LAMBRECHT, G., MUTSCHLER, E., BÄUMERT, H.G. & BURNSTOCK, G. (1993). PPADS selectivity antagonizes P_{2x}-purinoceptor-mediated responses in the rabbit urinary bladder. *Br. J. Pharmacol.*, **110**, 1491–1495.

ZIGANSHIN, A.U., HOYLE, C.H.V., LAMBRECHT, G., MUTSCHLER, E., BÄUMERT, H.G. & BURNSTOCK, G. (1994a). Selective antagonism by PPADS at P_{2x}-purinoceptors in rabbit isolated blood vessels. *Br. J. Pharmacol.*, **111**, 923–929.

ZIGANSHIN, A.U., HOYLE, C.H.V., ZIGANSHINA, L.E. & BURNSTOCK, G. (1994b). Effects of cyclopiazonic acid on contractility and ecto-ATPase activity in guinea-pig urinary bladder and vas deferens. *Br. J. Pharmacol.*, **113**, 669–675.

ZIYAL, R., PFAFF, O., WINDSCHEIF, U., BO, X., NICKEL, P., ARDANUY, U., BURNSTOCK, G., MUTSCHLER, E. & LAMBRECHT, G. (1994). A novel P_2-purinoceptor ligand which displays selectivity for the P_{2x}-subtype. *Drug Dev. Res.*, **31**, 336.

(Received June 17, 1994
Revised May 1, 1995
Accepted May 17, 1995)