Investigation of the effects of P2 purinoceptor ligands on the micturition reflex in female urethane-anaesthetized rats

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1 The effects of purinoceptor ligands for $P2X_1$ and/or $P2X_3$ receptors (α,β-meATP, IP_5I, TNP-ATP, MRS 2179, PPADS, Phenol red and RO116-6446/008; i.v., $n = 4$–5) and for $P2Y_1$ receptors (PPADS, MRS 2179 and MRS 2269; i.v., $n = 3$–5) were investigated on the distension-evoked 'micturition reflex' in the urethane-anaesthetized female rat.

2 α,β-meATP (180 nmol kg^{-1} min^{-1}), IP_5I (10, 30 and 100 nmol kg^{-1}), TNP-ATP (1 μmol kg^{-1}), MRS 2179 (1 μmol kg^{-1}) and PPADS (17 μmol kg^{-1}) each caused maintained bladder contractions to occur during the infusion of saline into the bladder. PPADS (17 μmol kg^{-1} min^{-1}) had a similar effect when infused intravesicularly. Regular bladder contractions were not observed until the infusion of saline was halted. For IP_5I, TNP-ATP, MRS 2179 and PPADS, the magnitude of postinfusion isovolumetric contractions was significantly reduced and, for IP_5I, this action was also associated with a significant reduction in urethral relaxation. Additionally, TNP-ATP caused a significant increase in the pressure and volume thresholds required to initiate a reflex.

3 Phenol red (a $P2X_1/P2X_3$ antagonist; 0.1 and 1 μmol kg^{-1}) caused a significant increase in the pressure and volume thresholds required to initiate a reflex and, at the higher dose, also caused a reduction in postinfusion isovolumetric contractions.

4 RO116-6446/008 (a $P2X_1$-selective antagonist; 1 and 10 μmol kg^{-1}) only caused a reduction in postinfusion isovolumetric contractions.

5 It is concluded that $P2X_1$ and $P2X_3$ receptors play a fundamental role in the micturition reflex in urethane-anesthetized female rats. $P2X_3$ receptor blockade raised the pressure and volume thresholds for the reflex, whereas $P2X_1$ receptor blockade diminished motor activity associated with voiding. $P2Y_1$ receptors may be involved in inhibition of rat detrusor tone.
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Abbreviations: MAP, mean arterial pressure; HR, heart rate; ATP, adenosine 5'-triphosphate; α,β-meATP, α,β-methylene ATP; PPADS, pyridoxal-5-phosphate-6-azophenyl 2',4'-disulphonic acid; IP_5I, diinosine pentaphosphate; EMG, electromyogram; EUS, external urethral sphincter; TP, threshold pressure

Introduction

The neural control of urinary bladder emptying involves many different transmitters: acetylcholine, monoamines (noradrenaline, dopamine and 5-hydroxytryptamine), amino acids (glutamate and GABA), various peptides (e.g. enkephalins and neurokinins) and nitric oxide (NO) (see De Groat & Yoshimura, 2001). Interestingly, NO seems to be more involved with urethral function rather than with the bladder (Wibberley *et al.*, 2002). Another transmitter system also identified as playing a role in urinary bladder contractions is the purinergic system, involving adenosine 5'-triphosphate (ATP) (Burnstock *et al.*, 1978). Although there is extensive literature (see Burnstock, 2001) to indicate that many different types of P2 purinoceptors are present in the lower urinary tract, the physiological role of these receptors in micturition (or voiding) is still uncertain. In part, this uncertainty has been

caused by a lack of P2 receptor subtype-selective agonists and antagonists to dissect the role of these receptors in the micturition reflex. To counteract the lack of selective receptor ligands for purinoceptors, gene knockout animal models have been used – particularly $P2X_1^{-/-}$, $P2X_2^{-/-}$ and $P2X_3^{-/-}$ gene deletion – in an attempt to identify the physiological roles of P2 receptor subtypes in the urinary bladder (Cockayne *et al.*, 2000; 2002; Vial & Evans, 2000; Vlaskovska *et al.*, 2001).

In recent years, a new generation of selective antagonists for P2 receptors has been developed and such agents now provide the means to test and extend observations gleaned from knockout animal models. Thus, the present study was carried out to investigate further the physiological roles of $P2X_1$, $P2X_3$ and $P2Y_1$ receptors in the micturition reflex in urethane-anaesthetized female rats using the archetypical smooth muscle P2X purinoceptor desensitizing agent α,β-methylene ATP (α,β-meATP; Kasakov & Burnstock, 1982), the P2X purinoceptor antagonist pyridoxal-5-phosphate-6-azophenyl

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$2',4'$-disulphonic acid (PPADS) (Lambrecht *et al.*, 1992) and also a number of compounds with some selectivity towards various purinoceptors, such as for $P2X_1$ receptors: diinosine pentaphosphate (IP_5I, also a $P2X_3$ receptor antagonist; King *et al*, 1999), trinitrophenyl adenosine $5'$-triphosphate (TNP-ATP, also a $P2X_3$ and $P2X_{2/3}$ receptor antagonist; Virginio *et al.*, 1998), RO116-6446/008 (a $P2X_1$ receptor antagonist; A.P. Ford, personal communication), Phenol red (phenolsulphonephthalein sodium, also a $P2X_3$ receptor antagonist; King *et al.*, 2003); and for $P2Y_1$ receptors: N^6-methyl $2'$-deoxyadenosine $3',5'$-bisphosphate (MRS 2179, also a $P2X_1$ receptor antagonist; Boyer *et al.*, 1998; Brown *et al.*, 2000), MRS 2269 (Boyer *et al*, 1998) and PPADS (usually considered a P2X receptor antagonist; see above). The micturition reflex was evoked by bladder distension induced by intravesicular infusion of saline, and measurements were made of reflex-evoked bladder and urethral pressures, as well as of external urethral sphincter (EUS) electromyography (EMG). Preliminary accounts of some of these findings have been published (Knowles *et al.*, 2000; 2001; 2003).

Methods

Experiments were carried out under the Animals (Scientific Procedures) Act, 1986. After completion of experiments, animals were killed by an overdose (administered i.v.) of pentobarbitone sodium.

General preparation

Experiments were carried out in 78 female, anaesthetized, spontaneously breathing Sprague–Dawley rats (250–350 g). Anaesthesia was induced and maintained during initial surgical procedures with isoflurane in oxygen (4%, reduced to 3%, as necessary). The left jugular vein, carotid artery and trachea were cannulated to permit intravenous injection of drugs, measurement of arterial blood pressure (and heart rate (HR)), and maintenance of a patent airway, respectively. Isoflurane administration was discontinued and anaesthesia was maintained for the remainder of each experiment with i.v. injections of urethane (25% solution, initial dose of 1.0–1.2 g kg^{-1}; see Maggi *et al.*, 1986). The depth of anaesthesia was assessed by the stability of blood pressure and HR, and by the absence of limb withdrawal in response to paw pinch. Supplementary doses of urethane (0.1 g kg^{-1}) were given when necessary. Blood pressure was measured using a pressure transducer (Gould Statham P23Db), and the HR was derived electronically on-line from the blood pressure signal using *AcqKnowledge version 3.5.3* software (Biopac Systems Inc., U.S.A.). Body core temperature was maintained between 36 and 38°C by feedback from a rectally placed thermosensor to a heated blanket. The animals were infused (6 ml kg^{-1} h^{-1}, i.v.) with a solution comprising 10 ml plasma substitute (*Gelofusine*; Braun Medical, Sheffield, U.K.), 10 ml distilled water, 0.04 g glucose and 0.168 g sodium bicarbonate, to prevent the development of nonrespiratory acidosis and to maintain blood volume.

Measurement of urinary bladder and urethral pressures and EUS-EMG

The ureters were isolated at the level of the kidney through retroperitoneal incisions. Each ureter was divided and the proximal end cannulated to drain the kidneys and prevent urine flow into the bladder. The rat's head was secured in a stereotaxic frame that was tilted at an angle of 10–20° so that the animal could lie in a supine position. This arrangement ensured that intravesical bladder pressure was not affected by the weight of the rat. The urinary bladder was exposed and two cannulae were inserted into the lumen of the bladder through an incision in the bladder dome. One cannula allowed the measurement of intraluminal pressure and the other permitted infusion of saline into the bladder. A third cannula was inserted through the bladder dome into the proximal urethra: for details of this method, plus a diagram, see Conley *et al.* (2001). The urethra was perfused at a constant rate (0.08 ml min^{-1}), so that changes in urethral pressure were reflected as changes in resistance to the flow of saline. For the measurement of external urethral striated muscle sphincter (EUS) EMG, two fine copper wire electrodes (0.2 mm diameter) were used and inserted percutaneously approximately 0.5 cm lateral and 0.5 cm caudal on each side of the external urethral opening from which the EMG signal was measured. It should be noted that such a measurement cannot preclude that activity observed is not from the pelvic floor muscle as well. At the end of experiments, animals were given decamethonium (3 mg kg^{-1}, i.v.) to confirm that the electrodes were in actuality recording from striated muscle EMG and also to determine the level of recording noise.

Experimental protocols

Surgical preparation was followed by a stabilization period of 30 min. During stabilization, inspired air was enriched with oxygen (0.05–0.10 L min^{-1}) and the blood gases and pH were monitored and maintained at 90–130 mmHg pO_2, 40–50 mmHg pCO_2 and pH 7.3–7.4. Saline was then infused into the urinary bladder at 0.05 ml min^{-1} (the maximal physiological rate of diuresis in the cat; see Klevmark, 1974), which resulted in a gradual increase in bladder wall muscle tension and, eventually, the occurrence of spontaneous phasic bladder contractions. The saline infusion was discontinued after three consecutive bladder contractions of the same amplitude. Bladder and urethral activities were monitored for a further 5 min before the saline was drained from the bladder through the bladder infusion cannula. This first infusion was carried out to 'test and prime' the system, and was followed by a rest period of 20 min. This priming procedure also ensured that the placement of the electrodes for recording the EMG signal was appropriate. A second infusion was carried out to evoke a 'control' reflex. Again, the saline infusion was discontinued after three consecutive bladder contractions of the same amplitude. Bladder and urethral activities were monitored for a further 5 min before the saline was drained from the bladder. After 5 min, either saline, vehicle (DMSO) or a test drug was injected (i.v.) or α, β-meATP infusion (i.v.) was begun and 5 min later a distension-induced bladder reflex was evoked as described above. In the case of the intravesicular infusion of PPADS, this was carried out after the control reflex. PPADS (10 mg ml^{-1}) was infused *via* the bladder cannula at a rate of 2 ml h^{-1} for 30 min. To prevent vesicular damage and the possibility of evoking micturition, the cannula measuring intravesicular pressure was open during this infusion period – thus allowing exposure of the bladder to PPADS and drainage of excess fluid. The

bladder was then fully drained and 5 min later a distension-induced bladder reflex was evoked as described above.

Data capture and analysis

Arterial blood pressures, bladder and urethral pressures were continuously displayed on a chart recorder (Grass Instruments) and captured (1500 samples s^{-1}) by an MP100WSW interface (Biopac Systems Inc., U.S.A.) to allow data to be acquired and analysed off-line using *AcqKnowledge version 3.5.3* software (Biopac Systems Inc., U.S.A.). HRs were derived electronically on-line from the blood pressure signal using this software. The amplified EMG signal was captured (1500 samples s^{-1}) and the input was integrated off-line, again using *AcqKnowledge*.

Analysis of reflex-evoked bladder and urethral responses and baseline values

The micturition reflexes, evoked before and after the test solution (i.v.), were selected for analysis (Figure 1). Saline infusion into the bladder evoked large-amplitude, rhythmic bladder contractions, which have been assumed to represent a micturition reflex (Maggi *et al.*, 1986). After saline infusion was discontinued, the mean amplitude (mmHg) and duration (s) of the next three bladder contractions were measured. These three bladder contractions were analysed because they represented isovolumetric reflex bladder contractions, that is, they occurred when the amount of saline in the bladder, and therefore resting bladder pressure, was constant. The mean amplitude (mmHg) and duration (s) of the three urethral relaxations accompanying isovolumetric bladder contractions were also measured. The mean area of the integrated EMG bursts associated with isovolumetric bladder contractions and urethral relaxations was measured as well. The micturition reflex pressure thresholds were taken as the bladder pressure (mmHg) at which the first reflex bladder contraction (i.e. the first fast rise in bladder pressure for those experiments in which a maintained contraction occurred) with concomitant reflex urethral activity (i.e. relaxation and high-frequency oscillations) occurred. The volume threshold (ml) was calculated from the time to evoke this first bladder contraction by the constant influx of saline infusion. All baseline values were the mean values over a 3 min period and were measured 2 min before reflexes and test agent responses. The following baseline measurements were made: urethral pressure and background activity (mean value measured over 3 min); mean arterial pressure (MAP) and HR (measured over 30 s, 2 min before the beginning of the infusion to evoke the control reflex). Changes in baseline variables caused by the test substances were measured 2 min before the test reflex and compared to the values obtained 2 min before the control reflex. It should be noted that the control reflex did not affect baseline values. Changes in these variables caused by either drugs or vehicle were measured as a percentage of the control values and compared using Student's unpaired *t*-test. Values of $P < 0.05$ were considered significant. All values are expressed as mean $\pm$ s.e.m. unless otherwise stated.

Drugs and solutions

Drugs and chemicals were obtained from the following sources: Phenol red, urethane and α,β-meATP (lithium salt) from Sigma Aldrich Chemical Co., (Poole, Dorset); pentobar-bitone sodium from Rhône Mérieux Ltd (Harlow, Essex); isoflurane from Abbott Labs (Queenborough, Kent); PPADS from Tocris Cookson Ltd (Bristol); TNP-ATP from Molecular Probes (Eugene, OR, U.S.A.); RO116-6446/008 (1*H*-benzo-imidazole-2-carboxylic acid[(*R*)-1-((1*S*,2*R*,3*S*)-1-cyclo-hexyl-methyl-3-cyclopropyl-2,3-dihydroxy-propylcarbamoyl)-2-thia-zol-4-yl-ethyl]-amide) was synthesized by Dr F. Padilla at Roche (Palo Alto, CA, U.S.A.); 2′-deoxy-N^6-methyladenosine 3′,5′-diphosphate diammonium (MRS 2179) and N^6-methyl-1,5-anhydro(2-adenin-9-yl)-2,3-dideoxy-D-arabino-hexitol-4′,6′-bisphosphate (MRS 2269) were gifts from Dr K.A. Jacobson at NIH (Bethesda, U.S.A.); IP$_5$I was a gift from Dr J. Pintor (University of Complutense, Madrid, Spain). Drugs were given i.v. as their respective salts. α,β-meATP, PPADS and IP$_5$I were dissolved in saline, while RO116-6446/008, MRS 2179, MRS 2269 and Phenol red were dissolved in 10% DMSO to give a final volume of administration of 0.1 ml.

Results

Initial reflex-evoked responses

Infusion of saline into the urinary bladder in 78 female rats caused distension of the bladder, which subsequently evoked a micturition reflex (Figure 1). This reflex was characterized by the appearance of rhythmic bladder contractions of 29 ± 3 mmHg. The contractions had a mean duration of 28 ± 5 s and occurred at a frequency of 1 ± 0.2 contractions min^{-1}. The mean bladder pressure threshold to evoke the micturition reflex was 8.2 ± 1.8 mmHg, which was reached when 0.10 ± 0.02 ml of saline had been infused into the bladder. In addition, each of the rhythmic bladder contractions was associated with a fall in urethral pressure of 16 ± 4 mmHg, which continued for 29 ± 3 s before returning to baseline levels. In 67 out of the 78 animals, high-frequency oscillations in urethral pressure occurred at the peak of each bladder contraction and these oscillations had a mean amplitude of 12 ± 4 mmHg and duration of 11 ± 3 s. In the remaining animals, high-frequency oscillations in urethral pressure were not observed. This was probably due to variations in the position of the urethral cannula. The fall in urethral pressure was accompanied by bursts of EUS-EMG activity. Each reflex bladder contraction was associated with an increase in MAP (6 ± 2 mmHg) and HR (16 ± 4 beats min^{-1}). The mean data for the control reflex in each experimental group are shown in Table 1. The combined mean baseline data for urethral pressures, MAP and HR were 20 ± 4 mmHg, 108 ± 2 mmHg and 391 ± 6 beats min^{-1}, respectively. The mean baseline data for individual experimental groups are shown in Table 2.

Effects of α,β-meATP (60 and 180 nmol kg^{-1} min^{-1}), PPADS (17 μmol kg^{-1}), IP$_5$I (10, 30 and 100 nmol kg^{-1}), TNP-ATP (1 μmol kg^{-1}) and MRS 2179 (0.1 and 1 μmol kg^{-1})

Over the period of continuous saline infusion into the bladder, the intravenous administration of each ligand caused the initial volume-evoked bladder contraction to be maintained, in contrast to the rhythmic contractions observed over the same time period in the control animals (Figures 2–6). These maintained contractions were associated with a maintained
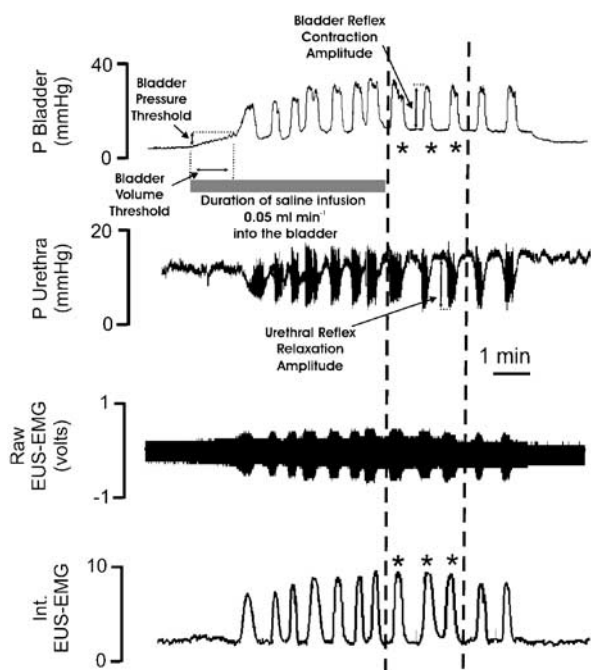
Figure 1 Analysis of bladder variables recorded from an urethane-anaesthetized female rat: traces showing the effects evoked by infusing ($0.5\,ml\,min^{-1}$) saline (stippled horizontal bar) into the bladder on bladder and urethra pressure (mmHg) and on raw and integrated external urethral sphincter (EUS-EMG) activity and how these changes were measured. Once three similar bladder contractions (phasic increases in bladder pressure) were observed, the saline infusion was switched off and measurements were made on the height and duration of each of these contractions (*), that is, those falling between the two dashed lines as well as the associated urethral relaxations and integrated EUS-EMG. These values were then averaged to give the control reflex-evoked changes (see Table 1). In addition, the thresholds, pressure and volume to evoke this reflex were also calculated. The measurements were made from the beginning of the saline infusion to the beginning of a bladder contraction associated with urethral relaxation.

fall in urethral pressure of between 7 and 10 mmHg and the appearance of sustained EUS-EMG activity. For α,β-meATP ($n = 5$), these effects were only observed at the dose of $180\,nmol\,kg^{-1}\,min^{-1}$ (Figure 2). The same effects were observed when PPADS (Figure 3) was administered either by intravenous injection ($17\,\mu mol\,kg^{-1}$, $n = 5$) or by intravesicular infusion ($17\,\mu mol\,kg^{-1}\,min^{-1}$, $n = 5$) and were also observed with Ip5I, TNP-ATP and MRS 2179 (i.v.).

For the postinfusion period when the bladder still contained saline, the actions of PPADS, IP5I, TNP–ATP and MRS 2179 differed from α,β-meATP in that the magnitude of iso-volumetric bladder contractions was significantly reduced by $21 \pm 5\%$ for i.v. PPADS (but not for PPADS given by intravesicular infusion), by 20 ± 5, 65 ± 8 and $81 \pm 5\%$, at the doses of 10, 30 (Figure 4) and $100\,nmol\,kg^{-1}$ for IP5I, by $10 \pm 2\%$ for TNP-ATP (Figure 5) and by $20 \pm 5\%$ for MRS 2179 (Figure 6) at the dose of $1\,\mu mol\,kg^{-1}$, respectively. With IP5I, the reduction in isovolumetric bladder contractions also correlated with significant decreases in the associated urethral relaxations (56 ± 11 and $79 \pm 12\%$) and increases in EUS-EMG activity (61 ± 11 and $72 \pm 13\%$). However, this was not observed for TNP-ATP and MRS 2179. TNP-ATP caused an increase in volume and pressure threshold (Figure 5) of

10 ± 2 and $12 \pm 2\%$, respectively. The combined data for all these ligands on reflex-evoked variables are shown in Table 3. None of these drugs affected baseline bladder or urethral pressures. Furthermore, these drugs also had little effect on blood pressure and HR. However, α,β-meATP, at both doses, and PPADS evoked a transient rise in MAP and, in the case of α,β-meATP, the infusion of this drug was associated with a transient fall in HR.

Effects of Phenol red (0.1 and $1\,\mu mol\,kg^{-1}$) on reflex-evoked responses

The P2X$_1$/P2X$_3$ antagonist Phenol red caused a significant increase in the pressure and volume thresholds required to evoke micturition compared with vehicle (see Table 3). A trace of one of the low-dose experiments is shown in Figure 7. At the higher dose, there was a significant reduction in the magnitude of isovolumetric bladder contractions by $15 \pm 9\%$, although neither dose affected the accompanying urethral relaxations and associated increase in EUS-EMG activity. Administration (i.v.) of Phenol red ($n = 5$) had no significant effect on the baseline bladder and urethral pressures, MAP and HR.

Effects of RO116-6446/008 (1 and $10\,\mu mol\,kg^{-1}$) on reflex-evoked responses

The P2X$_1$-selective antagonist RO116-6446/008 (i.v.) did not have any effect on the initial infusion-evoked reflex responses. However, the magnitude of isovolumetric bladder contractions was significantly reduced at both doses when compared to vehicle control (see Table 3). The associated reduction in urethral pressure and increase in EUS-EMG activity was similar to that seen with other P2X$_1$ antagonists (Figure 8), except at the higher dose where there was a small but significant inhibition of the increase in EUS-EMG activity. The volume and pressure thresholds required to evoke the micturition reflex were unaffected. RO116-6446/008 had no significant effect on the baseline bladder and urethral pressures, MAP and HR.

MRS 2269 ($1\,\mu mol\,kg^{-1}$) on reflex-evoked responses

Administration of the P2Y$_1$-selective antagonist MRS 2269 (i.v., $n = 3$) had no effect on the reflex (see Table 3) or baseline bladder and urethral pressures, MAP and HR.

Effects of vehicles on reflex-evoked responses and baseline values

Administration of the vehicles saline (bolus i.v. or slow infusion i.v.) or 10% DMSO (i.v.) had no significant effect on reflex-evoked changes in bladder and urethral pressures and EUS-EMG activity in all experimental groups (see Table 3). In addition, neither of the vehicles had any effect on baseline bladder and urethral pressures, blood pressure and HR.

Discussion

All the P2 purinoceptor ligands examined, with the exception of MRS 2269, affected the micturition reflex, indicating that

Table 1 Control reflex-evoked changes, caused by 'initial' bladder distension, in bladder and urethral pressures and urethral striated muscle activity (EUS-EMG) for each experimental group in urethane-anaesthetized female rats

Experimental group	n	Thresholds		Rhythmic bladder contractions		Urethral relaxations		EUS-EMG activity
		Bladder pressure (mmHg)	Saline volume (ml)	Amplitude (mmHg)	Duration (s)	Amplitude (mmHg)	Duration (s)	Total amount (area s^{-1})
Saline bolus i.v.	5	8.2 ± 1.8	0.10 ± 0.02	29 ± 3	28 ± 5	16 ± 4	29 ± 3	4.3 ± 0.5
Saline infusion i.v.	5	8.4 ± 1.5	0.12 ± 0.02	27 ± 5	27 ± 4	17 ± 5	29 ± 4	3.8 ± 0.9
DMSO	5	7.9 ± 1.4	0.12 ± 0.04	28 ± 4	29 ± 5	17 ± 3	31 ± 2	4.4 ± 0.7
α,β-meATP								
60 nmol kg^{-1} min^{-1}, i.v.	4	8.3 ± 2.1	0.10 ± 0.03	30 ± 4	27 ± 6	19 ± 5	30 ± 5	3.6 ± 0.9
180 nmol kg^{-1} min^{-1}, i.v.	5	8.2 ± 1.7	0.12 ± 0.05	31 ± 3	28 ± 2	16 ± 2	28 ± 3	4.3 ± 0.5
PPADS								
17 μmol kg^{-1}, i.v.	5	8.4 ± 1.5	0.13 ± 0.04	27 ± 3	29 ± 2	17 ± 5	30 ± 2	4.5 ± 0.6
17 μmol kg^{-1} min^{-1}	5	8.0 ± 2.2	0.11 ± 0.03	28 ± 2	28 ± 4	16 ± 3	29 ± 1	3.3 ± 0.8
IP$_5$I								
10 nmol kg^{-1}	3	7.7 ± 1.4	0.11 ± 0.05	30 ± 2	27 ± 3	18 ± 2	27 ± 4	3.6 ± 1.2
30 nmol kg^{-1}	5	8.6 ± 1.1	0.15 ± 0.01	27 ± 5	29 ± 4	16 ± 2	30 ± 3	3.9 ± 0.7
100 nmol kg^{-1}	5	8.2 ± 1.9	0.13 ± 0.03	28 ± 2	26 ± 5	18 ± 4	28 ± 1	4.1 ± 0.9
TNP-ATP								
1 μmol kg^{-1}	5	7.7 ± 1.6	0.19 ± 0.04	31 ± 2	30 ± 3	21 ± 5	30 ± 4	4.1 ± 1.2
MRS 2179								
0.1 μmol kg^{-1}	3	8.7 ± 1.5	0.13 ± 0.04	30 ± 6	26 ± 2	18 ± 2	29 ± 6	2.7 ± 1.1
1 μmol kg^{-1}	5	9.1 ± 1.3	0.12 ± 0.05	32 ± 3	28 ± 5	20 ± 3	29 ± 3	3.6 ± 0.8
Phenol red								
0.1 μmol kg^{-1}	5	8.1 ± 1.7	0.09 ± 0.03	28 ± 2	26 ± 3	19 ± 2	33 ± 2	4.2 ± 0.8
1 μmol kg^{-1}	5	7.6 ± 2.0	0.11 ± 0.04	32 ± 4	29 ± 4	17 ± 4	35 ± 3	3.7 ± 0.7
RO116-6446/008								
1 μmol kg^{-1}	5	7.8 ± 1.5	0.09 ± 0.03	30 ± 1	26 ± 5	17 ± 3	27 ± 2	3.2 ± 0.4
10 μmol kg^{-1}	5	7.6 ± 1.3	0.08 ± 0.02	30 ± 4	24 ± 2	19 ± 2	25 ± 1	3.8 ± 0.8
MRS 2269								
1 μmol kg^{-1}	3	8.9 ± 1.9	0.08 ± 0.02	30 ± 4	28 ± 4	15 ± 3	30 ± 2	3.3 ± 1.4

All values are mean $\pm$ s.e.m.

Table 2 Baseline values, before 'initial' bladder distension-evoked reflexes, for urethral MAP and HR for each experimental group in urethane-anaesthetized female rats

Experimental group	n	Bladder pressure (mmHg)	Urethral pressure (mmHg)	MAP (mmHg)	Heart (beat)
Saline bolus i.v.	5	2.9 ± 0.4	20.2 ± 3.2	102 ± 4	385 ± 10
Saline infusion i.v.	5	2.4 ± 0.5	18.1 ± 2.7	110 ± 3	376 ± 12
DMSO i.v.	5	3.3 ± 0.4	23.3 ± 2.2	109 ± 3	401 ± 12
α,β-meATP					
60 nmol kg^{-1} min^{-1}, i.v.	4	2.3 ± 0.8	19.6 ± 2.8	112 ± 5	390 ± 8
180 nmol kg^{-1} min^{-1}, i.v.	5	3.7 ± 0.6	21.5 ± 4.6	108 ± 2	395 ± 15
PPADS					
17 μmol kg^{-1}, i.v.	5	2.7 ± 0.6	18.6 ± 3.2	115 ± 5	380 ± 18
17 μmol kg^{-1} min^{-1}	5	2.4 ± 0.5	21.1 ± 4.6	112 ± 2	389 ± 13
IP$_5$I					
10 nmol kg^{-1}	3	3.2 ± 0.9	19.6 ± 5.3	108 ± 4	374 ± 14
30 nmol kg^{-1}	5	2.8 ± 0.5	17.3 ± 2.4	107 ± 2	393 ± 9
100 nmol kg^{-1}	5	3.5 ± 0.6	19.1 ± 4.3	115 ± 3	386 ± 12
TNP-ATP					
1 μmol kg^{-1}	5	3.4 ± 0.5	22.5 ± 5.9	120 ± 8	383 ± 12
MRS 2179					
0.1 μmol kg^{-1}	3	3.8 ± 0.9	17.8 ± 5.6	104 ± 6	393 ± 14
1.0 μmol kg^{-1}	5	2.7 ± 0.7	20.9 ± 2.6	118 ± 3	375 ± 10
Phenol red					
0.1 μmol kg^{-1}	5	2.6 ± 0.5	18.2 ± 4.6	112 ± 5	404 ± 20
1 μmol kg^{-1}	5	2.5 ± 0.8	19.2 ± 2.8	116 ± 4	377 ± 18
RO116-6446/008					
1 μmol kg^{-1}	5	2.2 ± 0.6	18.3 ± 4.4	106 ± 3	387 ± 8
10 μmol kg^{-1}	5	2.5 ± 0.5	16.4 ± 3.5	101 ± 5	382 ± 14
MRS 2269					
1 μmol kg^{-1}	3	3.1 ± 1.1	21.1 ± 4.2	113 ± 5	390 ± 7

All values are mean $\pm$ s.e.m.
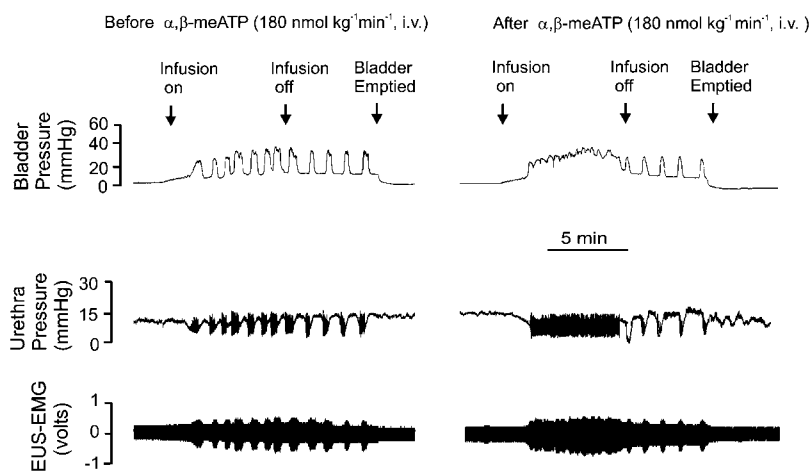
Figure 2 Urethane-anaesthetized female rat: traces showing the effects of i.v. infusion of α,β-meATP ($180\,\mathrm{nmol\,kg^{-1}\,min^{-1}}$) for 5 min on bladder and urethral pressure (mmHg) and on raw external urethral sphincter (EUS-EMG) activity during the micturition reflex evoked by distension of the urinary bladder by saline ($0.05\,\mathrm{ml\,min^{-1}}$). The first panel shows the control effects of infusion of saline into the bladder (infusion on) to evoke contractions (increases in bladder pressure); after three similar contractions the infusion was stopped (infusion off) and the bladder allowed to contract isovolumetrically. At 5 min after the saline infusion had been switched off, the bladder was emptied. The second panel shows the same scenario 5 min after infusion of α,β-meATP. Note that there were no rhythmic contractions during the infusion of saline and this was associated with a maintained relaxation of the urethra and an increase in EUS-EMG activity, but these rhythmic contractions of the bladder returned on switching off the saline infusion.
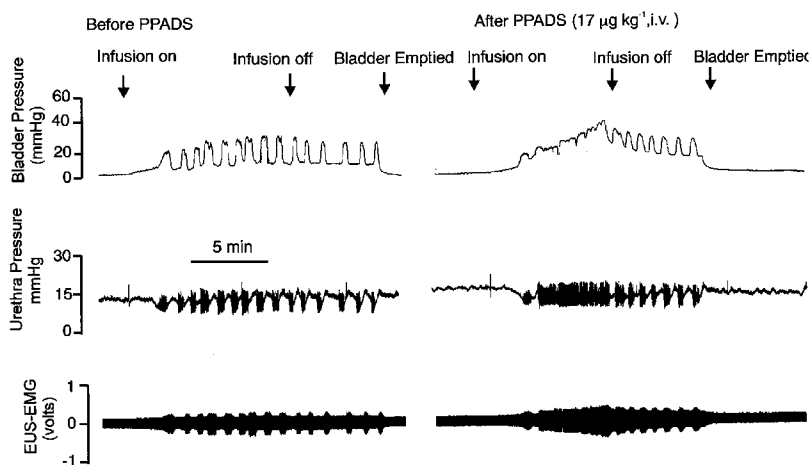
Figure 3 Urethane-anaesthetized female rat: traces showing the effects of i.v. PPADS ($17\,\mu\mathrm{mol\,kg^{-1}}$) on bladder and urethra pressure (mmHg) and on raw external urethral sphincter (EUS-EMG) activity during the micturition reflex evoked by distension of the urinary bladder by saline ($0.05\,\mathrm{ml\,min^{-1}}$). The first panel shows the control effects of infusion of saline into the bladder (infusion on) to evoke contractions (increases in bladder pressure); after three similar contractions the infusion was stopped (infusion off) and the bladder allowed to contract isovolumetrically. At 5 min after the saline infusion had been switched off, the bladder was emptied. The second panel shows the same scenario 5 min after administration i.v. of PPADS. Note that there were no rhythmic contractions during the infusion of saline and this was associated with a maintained relaxation of the urethra and increase in EUS-EMG activity but these rhythmic contractions of the bladder returned on switching off the saline infusion.

P2 purinoceptors do play a physiological role in the control of micturition, at least in the rat. These effects were mainly concerned with bladder regulation – rather than urethral regulation – and could be divided into three phenomena: increasing the volume and pressure thresholds for initiation of the reflex bladder contractions; attenuation of the height of the isovolumetric contractions after saline infusion was terminated; replacement of rhythmic contractions with maintained contractions during the distension period. TNP-ATP and Phenol red were the only ligands found to increase significantly the volume and pressure thresholds to elicit micturition. In the following potency order (based on the threshold concentration to cause inhibition) IP$_5$I, TNP-ATP, RO116-6446/008, Phenol red, MRS 2179 and PPADS caused a significant attenuation of the height of the isovolumetric contractions. Curiously, the slow infusion (i.v.) of α,β-meATP did not affect the height of postinfusion isovolumetric bladder contractions. Lastly, all the tested ligands (except RO116-6446/008 and Phenol red) caused maintained contractions of the bladder during the distension period. The potency order (based on the threshold concentration to elicit tonic contraction) was IP$_5$I, TNP-ATP, MRS 2179, followed by PPADS; α,β-meATP also caused maintained contractions although it acts primarily as a desensitizing agonist/antagonist (Kasakov & Burnstock, 1982). Thus, the
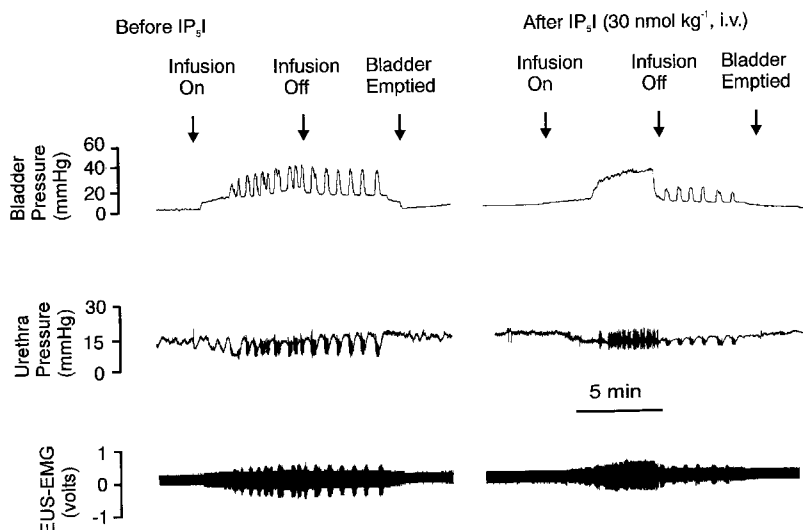
Before IP₅I

After IP₅I (30 nmol kg⁻¹, i.v.)

Figure 4 Urethane-anaesthetized female rat: traces showing the effects of i.v. IP₅I (30 nmol kg⁻¹) on bladder and urethra pressure (mmHg) and on raw external urethral sphincter (EUS-EMG) activity during the micturition reflex evoked by distension of the urinary bladder by saline (0.05 ml min⁻¹). The first panel shows the control effects of infusion of saline into the bladder (infusion on) to evoke contractions (increases in bladder pressure); after three similar contractions the infusion was stopped (infusion off) and the bladder allowed to contract isovolumetrically. At 5 min after the saline infusion had been switched off, the bladder was emptied. The second panel shows the same scenario 5 min after administration i.v. of IP₅I. Note that there were no rhythmic contractions during the infusion of saline and this was associated with a maintained relaxation of the urethra and increase in EUS-EMG activity. On turning off the saline infusion, these rhythmic bladder contractions returned although of reduced magnitude.
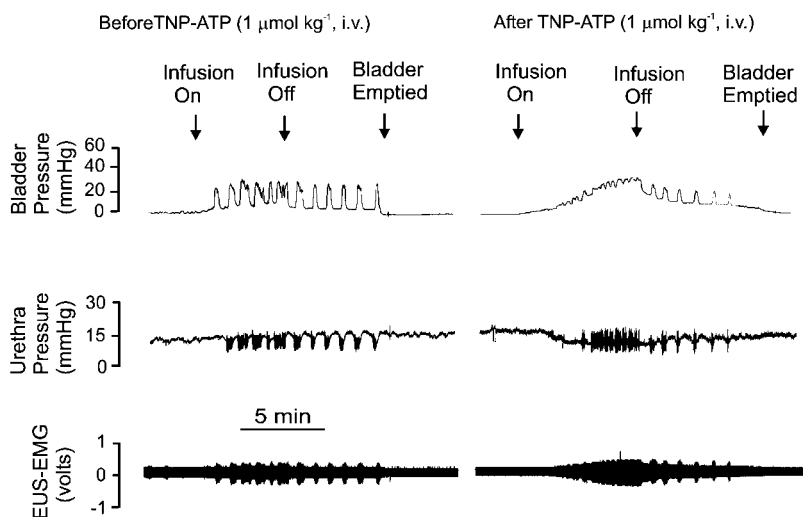
BeforeTNP-ATP (1 μmol kg⁻¹, i.v.)

After TNP-ATP (1 μmol kg⁻¹, i.v.)

Figure 5 Urethane-anaesthetized female rat: traces showing the effects of i.v. TNP-ATP (1 μmol kg⁻¹) on bladder and urethra pressure (mmHg) and on raw external urethral sphincter (EUS-EMG) activity during the micturition reflex evoked by distension of the urinary bladder by saline (0.05 ml min⁻¹). The first panel shows the control effects of infusion of saline into the bladder (infusion on) to evoke contractions (increases in bladder pressure); after three similar contractions the infusion was stopped (infusion off) and the bladder allowed to contract isovolumetrically. At 5 min after the saline infusion had been switched off, the bladder was emptied. The second panel shows the same scenario 5 min after administration i.v. of TNP-ATP. Note that there were no rhythmic contractions during the infusion of saline and this was associated with a maintained relaxation of the urethra and increase in EUS-EMG activity. On turning off the saline infusion, these rhythmic bladder contractions returned although of reduced magnitude.

question arose, by what mechanisms did these P2 ligands bring about the above three phenomena?

Increases in volume and pressure thresholds

For the first of these phenomena (increase in the micturition threshold), it is suggested that the key to the observed changes can be found in the nature of the neurogenic reflex evoked by saline infusion into the urinary bladder. The steady infusion of saline and subsequent increase in wall smooth muscle tension will activate the sensory nerves of the urinary bladder (Maggi *et al*., 1986). The suburothelial sensory nerve endings of the rat bladder are heavily invested with P2X₃ receptors (Cockayne *et al*., 2000; Elneil *et al*., 2001; Vlaskovska *et al*., 2001) and the
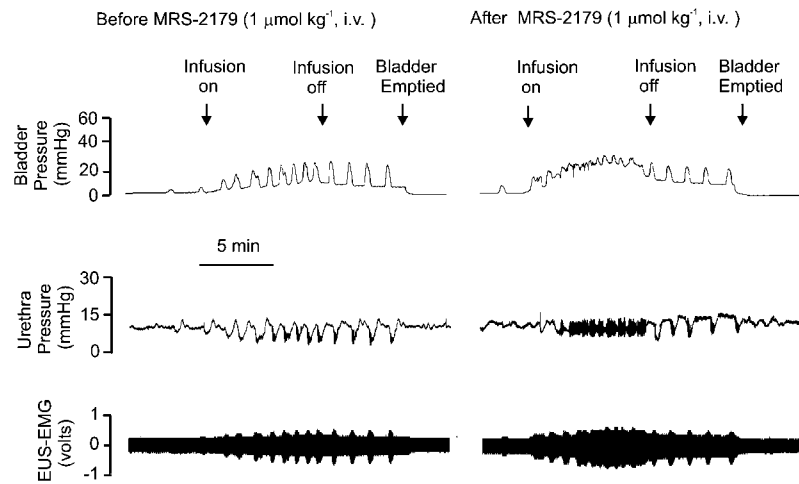
Figure 6 Urethane-anaesthetized female rat: traces showing the effects of i.v. MRS 2179 (1 μmol kg^{-1}) on bladder and urethra pressure (mmHg) and on raw external urethral sphincter (EUS-EMG) activity during the micturition reflex evoked by distension of the urinary bladder by saline (0.05 ml min^{-1}). The first panel shows the control effects of infusion of saline into the bladder (infusion on) to evoke contractions (increases in bladder pressure); after three similar contractions the infusion was stopped (infusion off) and the bladder allowed to contract isovolumetrically. At 5 min after the saline infusion had been switched off, the bladder was emptied. The second panel shows the same scenario 5 min after administration i.v. of MRS 2179. Note that there were no rhythmic contractions during the infusion of saline and this was associated with a maintained relaxation of the urethra and increase in EUS-EMG activity. On turning off the saline infusion, these rhythmic bladder contractions returned although of reduced magnitude.

Table 3 Changes (Δ) in % from control reflex-evoked increases in bladder pressure, urethral relaxation, increase in urethral striated muscle activity (EUS-EMG) and the pressure threshold to initiate these reflex-evoked effects caused by test substances in urethane-anaesthetized female rats

Experimental group	n	Δ Reflex-evoked bladder contraction (%)	Δ Reflex-evoked urethral relaxations (%)	Δ Reflex-evoked EUS-EMG (%)	Δ Reflex pressure threshold (%)
Saline bolus i.v.	5	-3 ± 2	-4 ± 7	-4 ± 4	-6 ± 5
Saline infusion i.v.	5	-9 ± 3	-4 ± 3	-3 ± 2	-7 ± 3
DMSO i.v.	5	-2 ± 2	-6 ± 3	3 ± 6	-6 ± 4
α,β-meATP					
60 nmol kg^{-1}, i.v.	4	-4 ± 2	-8 ± 2	-4 ± 3	-1 ± 2
180 nmol kg^{-1} min^{-1}, i.v.	5	-8 ± 5	-2 ± 3	-5 ± 3	-5 ± 3
PPADS					
17 μmol kg^{-1}, i.v.	5	-21 ± 5*	6 ± 8	-3 ± 4	-4 ± 3
17 μmol kg^{-1} min^{-1}	5	-9 ± 5	5 ± 3	5 ± 2	-4 ± 2
IP$_5$I					
10 nmol kg^{-1}	3	-20 ± 5*	-7 ± 3	-13 ± 9	-3 ± 5
30 nmol kg^{-1}	5	-65 ± 8**	-56 ± 3**	-61 ± 11**	-8 ± 3
100 nmol kg^{-1}	5	-81 ± 5**	-79 ± 11**	-72 ± 13**	-5 ± 5
TNP-ATP					
1 μmol kg^{-1}	5	-10 ± 2*	-2 ± 2	-2 ± 2	12 ± 2*
MRS 2179					
0.1 μmol kg^{-1}	3	-5 ± 2	-3 ± 3	2 ± 2	-4 ± 2
1.0 μmol kg^{-1}	5	-20 ± 5*	7 ± 3	-13 ± 9	-5 ± 5
Phenol red					
0.1 μmol kg^{-1}	5	-5 ± 3	-4 ± 2	-10 ± 4	84 ± 9**
1 μmol kg^{-1}	5	-15 ± 9*	-6 ± 3	-8 ± 4	60 ± 3**
RO116-6446/008					
1 μmol kg^{-1}	5	-18 ± 3*	18.3 ± 4.4	-7 ± 4	2 ± 2
10 μmol kg^{-1}	5	-26 ± 8**	$16.4 \pm \pm 3.5$	-10 ± 4	4 ± 6
MRS 2269					
1 μmol kg^{-1}	3	-5 ± 2	-5 ± 3	-11 ± 4	-3 ± 5

All values are mean $\pm$ s.e.m. *$P < 0.05$; **$P < 0.001$ compared with the appropriate vehicle control using Student's unpaired t-test.

cell bodies of afferent nerves passing through rat bladder smooth muscle also possess P2X$_3$ receptors as well as heteromeric P2X$_{2/3}$ receptors (Zhong *et al.*, 2003). The urothelium itself releases ATP in proportion to the intravesi-cular pressure and thereby activates P2X receptors on bladder sensory nerves (Ferguson *et al.*, 1997; Vlaskovska *et al.*, 2001). Deletion of the P2X$_3$ gene (P2X$_3^{-/-}$) results in a marked urinary bladder hyporeflexia, in terms of increased voiding volume

(Cockayne *et al.*, 2000) and markedly decreased bladder afferent sensitivity in response to increasing intravesicular pressure (Vlaskovska *et al.*, 2001). Furthermore, deletion of the $P2X_2$ gene ($P2X_2^{-/-}$), and loss of heteromeric $P2X_{2/3}$ and homomeric $P2X_2$ receptors, also results in a marked urinary bladder hyporeflexia, in terms of increased thresholds to contraction on bladder filling (Ford *et al.*, 2002).

From the above observations, it could be predicted that those P2 purinoceptor ligands showing blocking activity at homomeric $P2X_3$ receptors and heteromeric $P2X_{2/3}$ receptors (see Table 4) would significantly alter the volume and pressure

thresholds of the micturition reflex. In this respect, TNP-ATP did alter the volume and pressure thresholds in our experiments (Figure 5). This drug is a potent inhibitor of cloned rat $P2X_3$ and $P2X_{2/3}$ receptors (Virginio *et al.*, 1998; Liu *et al.*, 2001) and native rat $P2X_3$ and $P2X_{2/3}$ receptors in isolated DRG neurons (Burgard *et al.*, 1999). It is also active under *in vivo* conditions and, in comparison to morphine, blocked nociceptive behaviour to the same extent in mice (Honore *et al.*, 2002). The actions of TNP-ATP on the volume and pressure thresholds were mimicked by Phenol red (Figure 7) – a newly identified P2 purinoceptor ligand (King *et al.*, 2003) – which is
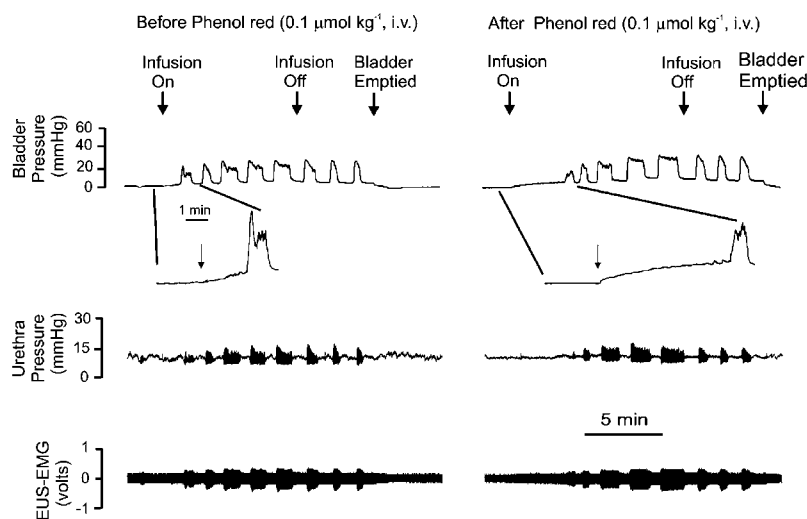

Figure 7 Urethane-anaesthetized female rat: traces showing the effects of i.v. Phenol red ($0.1 \, \mu mol \, kg^{-1}$) on bladder and urethra pressure (mmHg) and on raw external urethral sphincter (EUS-EMG) activity during the micturition reflex evoked by distension of the urinary bladder by saline ($0.05 \, ml \, min^{-1}$). The first panel shows the control effects of infusion of saline into the bladder (infusion on) to evoke contractions (increases in bladder pressure); after three similar contractions the infusion was stopped (infusion off) and the bladder allowed to contract isovolumetrically. At 5 min after the saline infusion had been switched off, the bladder was emptied. There is also an inset showing an expanded time base of the period it took for the saline infusion to initiate a bladder contraction. The second panel shows the same scenario 5 min after administration i.v. of the low dose of phenol red. It should be noted that the time to initiate the first bladder contraction by the saline infusion was delayed. This is better illustrated by comparing the expanded time base inserted with that in the control panel.
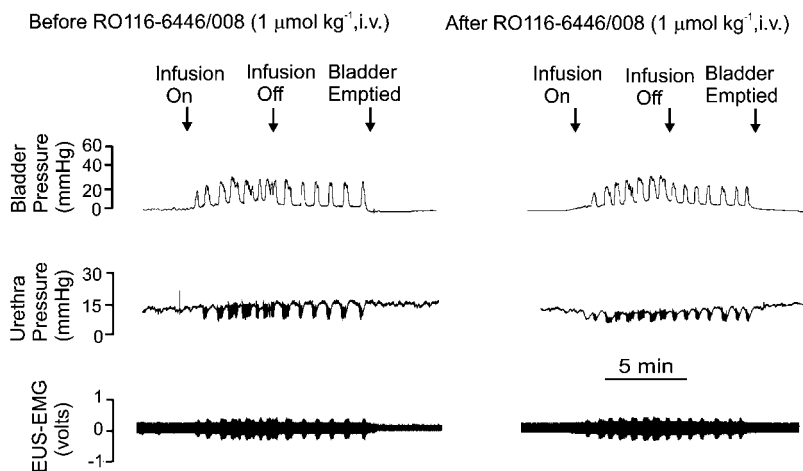

Figure 8 Urethane-anaesthetized female rat: traces showing the effects of i.v. RO116-6446/008 ($1 \, \mu mol \, kg^{-1}$) on bladder and urethra pressure (mmHg) and on raw external urethral sphincter (EUS-EMG) activity during the micturition reflex evoked by distension of the urinary bladder by saline ($0.05 \, ml \, min^{-1}$). The first panel shows the control effects of infusion of saline into the bladder (infusion on) to evoke contractions (increases in bladder pressure); after three similar contractions the infusion was stopped (infusion off) and the bladder allowed to contract isovolumetrically. At 5 min after the saline infusion had been switched off, the bladder was emptied. The second panel shows the same scenario 5 min after administration i.v. of RO116-6446/008.

the least potent of the tested drugs at recombinant P2X$_3$ receptors and is quite inactive at P2X$_{2/3}$ receptors (Table 4). Phenol red mimicry of TNP-ATP, yet its inactivity at P2X$_{2/3}$ receptors, suggested that TNP-ATP could not exert its actions *via* P2X$_{2/3}$ receptors. Instead, TNP-ATP and phenol red may have acted solely on P2X$_3$ receptors. The higher potency *in vivo* of non-nucleotidic phenol red compared to other tested P2X$_3$ receptor antagonists (including PPADS) might be explained by its resistance to ectoATPase breakdown (as opposed to the nucleotide-based antagonists used) and that it does not possess a chemically unstable azo bridge (in contrast to PPADS). Accordingly, its relative potency may well be higher *in vivo* than so far noted *in vitro*. Thus, the weight of evidence suggests that only those drugs blocking P2X$_3$ receptors significantly altered the threshold of the micturition reflex, a conclusion supported by the observed disruption of urinary bladder reflexes in P2X$_3^{-/-}$ gene-deficient mice (Cockayne *et al.*, 2000; Vlaskovska *et al.*, 2001).

Attenuation of isovolumetric contractions

The second major action of the drugs tested was the attenuation of the height of the isovolumetric phasic contractions in the bladder after saline infusion was terminated. The motor innervation of the rat detrusor muscle is complex and involves both cholinergic and purinergic components (see Burnstock, 2001). It has been shown that atropine-resistant (non-cholinergic) electrical events (EJPs) underpinning phasic contractions are purinergic in rat detrusor muscle (Hoyle & Burnstock, 1985). Also, arterial injections of ATP and α, β-meATP close to the bladder immediately initiate phasic contractions in the unanaesthetized rat (Igawa *et al.*, 1993). Furthermore, the P2X receptor antagonist PPADS inhibited purinergic motor responses in the pithed rat (Hegde *et al.*, 1998), and the P2 receptor antagonists suramin and PPADS displaced high-affinity [^{3}H]-α,β-meATP binding from rat detrusor muscle preparations (Bo *et al.*, 1994). Additionally, there is dense P2X$_1$-like immunoreactivity (Hansen *et al.*, 1998; Lee *et al.*, 2000; Elneil *et al.*, 2001) in close apposition to motor nerve varicosities in the rat detrusor (Hansen *et al.*, 1998), and there is a significant reduction in neurogenic motor activity in the bladder of P2X$_1^{-/-}$ gene-deficient mice (Vial & Evans, 2000).

The above observations on the motor innervation of the bladder are consistent with the involvement of P2X receptors, most likely the P2X$_1$ receptor subtype. In the present study, the observed potency order for P2 purinoceptor ligands attenuating phasic bladder contractions, *in vivo*, was in good agreement with their potency order for blockade of cloned rat P2X$_1$ receptors, *in vitro* (see Table 4). This list of P2X$_1$-active drugs included PPADS and MRS 2179, each of which is also known to block P2Y$_1$ receptors (Table 4). Since this G-protein-coupled P2Y$_1$ receptor has been located in rat detrusor (Obara *et al.*, 1998), the selective P2Y$_1$ receptor antagonist MRS 2269 (Nanadanan *et al.*, 1999; Brown *et al.*, 2000) was tested but was found to be inactive. Thus, again the weight of evidence suggested that the observed reduction in isovolumetric phasic contractions was due to an effective blockade of P2X$_1$ receptors in rat detrusor muscle, and that the residual phasic contractions after administration of P2X$_1$ receptor antagonists were in all probability cholinergic in origin.

Maintained urinary bladder contraction

The third major action of the tested compounds – but not of Phenol red and RO116-6446/008 at the doses given – was the maintained bladder contraction after the threshold for the micturition reflex had been reached. The inactivity of Phenol red and RO116-6446/008, which are P2X$_1$ receptor antagonists, suggested that maintained bladder contraction could not be mediated by the P2X$_1$ subtype. Of the other tested drugs, the most potent was IP$_5$I followed by TNP-ATP, MRS 2179 and PPADS in this order. Curiously, the desensitizing agonist/antagonist α,β-meATP also mimicked the above blocking agents. No single P2X or P2Y receptor is blocked by this range of antagonists apart from the P2X$_1$ subtype, but, even then, it is difficult to imagine how blockade of excitatory P2X$_1$ receptors could lead to maintained contraction of the bladder. Thus, it is proposed that these maintained contractions may involve the blockade of other types of P2 purinoceptors distinct from P2X$_1$.

The ability of MRS 2179 (and PPADS) to cause maintained contraction implies an involvement of P2Y$_1$ receptors, which are known to be present in the rat detrusor (Obara *et al.*, 1998). In carbachol-precontracted rat and mouse detrusor muscle, muscle tone is inhibited by ATP (Boland *et al.*, 1993;

Table 4 Potency of P2 purinoceptor ligands at recombinant P2X$_1$, P2X$_{2/3}$, P2X$_3$ (rat (r) isoforms) and P2Y$_1$ (turkey (t) isoform) receptors

	rP2X$_3$ receptor[a,b,c,d] *EC$_{50}$/IC$_{50}$ (μM)	*rP2X$_{2/3}$ receptor*[a,c] *EC$_{50}$/IC$_{50}$ (μM)	*rP2X$_1$ receptor*[b,e,f] *EC$_{50}$/IC$_{50}$ (μM)	*tP2Y$_1$ receptor*[f,g] IC$_{50}$/K$_b$[§] (μM)
α,β-meATP[a,g]	*1.9±0.3	*8.6±1.2	*3.2±0.2	Inactive
TNP-ATP[a,e]	0.29±0.04 (×10^{-3})	11.2±2.5 (×10^{-3})	1.1±0.04 (×10^{-3})	ND
PPADS[a,e,g]	0.22±0.05	2.3±0.4	0.12±0.07	1.58[§]
IP$_5$I[a,e]	2.8±0.7	Inactive	3.1±0.4 (×10^{-3})	ND
MRS 2179[b,f]	12.9±0.1	ND	1.15±0.21	0.33±0.06
Phenol red[c]	25.2±0.3	Inactive	3.3±0.3	ND
RO116-6446/008[d]	Inactive at 1 μM	ND	0.97±0.07 (×10^{-3})	ND
MRS 2269[b,f]	Inactive at 10 μM	ND	Inactive at 10 μM	1.64±0.43

EC$_{50}$ (*: for α,β-meATP) and either IC$_{50}$ or K$_b$ (§) values for other drugs acting at recombinant P2 receptors. Data expressed as mean±s.e.m. and taken from: [a]Liu *et al.* (2001); [b]Brown *et al.*(2000); [c]King *et al.* (2003); [d]B.F. King, J. Gever & A.P. Ford (unpublished data); [e]Wildman *et al.* (2002); [f]Nandanan *et al.* (1999); [g]Schachter *et al.* (1996), [h]Dunn *et al.* (2000).

Bolego *et al.*, 1995) through a G-protein-coupled P2Y$_1$-like receptor that is unaffected by adenosine receptor antagonists and adenosine uptake inhibitors (Bolego *et al.*, 1995). An inhibitory G-protein-coupled P2Y$_1$-like receptor has also been found in marmoset detrusor, where inhibition was evoked by ATP = 2-MeSATP ⩾ ADP (α,β-meATP inactive) and was unaffected by adenosine receptor antagonists (McMurray *et al.*, 1998). If inhibitory P2Y$_1$ receptors occur in rat detrusor, as the above relaxant actions of ATP, 2-MeSATP and ADP suggest, the maintained contractions caused by MRS 2179 (and PPADS) might be due to the blockade of P2Y$_1$ receptors excited by ATP released from the distension-stimulated urothelium. The inactivity of the P2Y$_1$-selective antagonist MRS 2269 ran counter to a proposal for inhibitory P2Y$_1$ receptors, unless the dose used was too low to cause an effective blockade. MRS 2269 is five-fold less potent than MRS 2179 at turkey P2Y$_1$ receptors (Table 4), and could not be used at a five-fold higher dose in our experiments because only a small sample of this development compound was available.

The role of IP$_5$I and TNP-ATP (and α,β-meATP) in also causing maintained contraction remains unclear. None of these drugs is known to block P2Y$_1$ receptors and, therefore, they must have exerted their effect in another way. IP$_5$I and TNP-ATP are antagonists of the P2X$_3$ receptors on sensory nerve endings beneath the urothelium. TNP-ATP, but not IP$_5$I, blocks heteromeric P2X$_{2/3}$ receptors and, therefore, IP$_5$I mimicry of TNP-ATP suggested that P2X$_{2/3}$ receptors were not involved. Therefore, it is possible that blockade of P2X$_3$ receptors by TNP-ATP and IP$_5$I (and by the desensitizing agent α,β-meATP) might prevent local axon reflexes from releasing ATP to activate inhibitory P2Y$_1$ receptors. Certainly, sensory nerve endings are known to release ATP (Holton, 1959) and local axon reflexes are also known to occur within the detrusor muscle (Lecci *et al.*, 1999). Thus, this further proposal of local axon reflexes, combined with the presence of inhibitory P2Y$_1$ receptors, could possibly explain the complex pharmacology of P2 purinoceptor antagonists in causing maintained contraction of the bladder.

Conclusion

It should be pointed out that the above interpretations of these purinoceptor ligands have considered that the main site of action is in the periphery, at the level of bladder. However, it is possible that some of the differences in the actions between these ligands could be due to these drugs having the ability to access different sites at which purinoceptors are involved in micturition, for instance the central nervous system. Little is known about central purinergic pathways in the control of micturition, if it is involved at all, and the present experiments with ionically charged antagonists that might not easily pass through the blood–brain barrier do tend to favour a peripheral site of action for the effects of these ligands. In this latter case, the ability to cause a maintained bladder contraction after the threshold for the micturition reflex has been reached seems to be peripheral, at the level of bladder itself, as PPADS also causes this effect when applied intravesically. Another surprising observation was that when isovolumetric bladder contractions were attenuated, as in the case of PPADS, TNP-ATP and MRS 2179, there was no reduction in the associated urethral relaxation. Such relaxations were only observed for IP$_5$I, and the mechanism behind this observation remains to be determined.

Thus, in summary, P2 purinoceptor ligands interacting with P2X$_3$ receptors appeared to alter the initiation of the micturition reflex, whereas P2 ligands interacting with P2X$_1$ receptors altered the phasic contractions associated with voiding. Additionally, P2Y$_1$ receptor blockade may remove an accommodatory, inhibitory drive to rat detrusor muscle. These *in vivo* experiments demonstrate the importance of purinergic signalling in the urinary bladder, at least in the rat, and further indicate that the development of drugs selective to each P2 purinoceptor subtype may be influential in correcting urinary bladder instability in its many clinical forms.

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