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The biphasic response of rat vesical smooth muscle to ATP

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1 Adenosine-5'-triphosphate (ATP) is known to exert a variety of biological effects via the activation of either ionotropic P_{2X}- or G-protein coupled P_{2Y}-purinoceptor subtypes. In this study the effects induced by ATP and ATP analogues on rat bladder strips were characterized at resting tone and in carbachol-prestimulated tissues.

2 ATP exerted a clear concentration-dependent biphasic response, which was maximal at 1 mM concentration and was characterized by an immediate and transient contraction, followed by a slower sustained relaxation. The receptor mediating contraction was susceptible to desensitization by ATP and by the ATP analogue, α,β-methyleneATP (α,β-meATP) showing the typical features of the P_{2X}-purinoceptor; conversely, ATP-evoked relaxation did not undergo tachyphylaxis following either ATP or α,β-meATP.

3 The slower and sustained relaxant phase seemed to be due to activation of P_{2Y}-purinoceptors, based on responses obtained with the P_{2Y} agonist, 2-methyl-thioATP (2-meSATP) and, more importantly, based on the clear involvement of the G-proteins. In fact, the G-protein activator, guanosine 5'-O-(3-thiotriphosphate) (GTPγS) significantly potentiated and the G-protein blocking agent, guanosine 5'-O-(2-thio-diphosphate) (GDPβS) completely abolished the ATP-induced relaxation. No effects were exerted by these two G-protein modulators on the ATP-induced contraction.

4 The relaxant component of the ATP response of bladder tissue was not significantly influenced by nitro-benzyl-thioinosine (NBTI) or by 8-phenyltheophylline (8-PT), suggesting that the contribution of the ATP metabolite adenosine to this response was negligible. Moreover, relaxation evoked by ATP and by the adenosine analogue, 5'-N-ethylcarboxamidoadenosine (NECA) was additive.

5 Suramin was unable to modify either the relaxant or the contractile responses of bladder strips to ATP. However, when tested on the concentration-response curve to the slowly hydrolysable P_{2X}-agonist α,β-meATP, a rightward shift was detected, suggesting that ATP contractile responses are mediated by suramine-sensitive P_{2X}-purinoceptors.

6 Uridine-5'-triphosphate (UTP) only induced a rapid and concentration-dependent contraction of the rat bladder preparation, which was not desensitized by pre-exposure to α,β-meATP, suggesting that UTP responses were not mediated by the 'classical' P_{2X}-purinoceptor.

7 It is therefore concluded that both P_{2X}- and P_{2Y}-purinoceptors, which mediate ATP-induced contraction and relaxation, respectively, are present in rat bladder. Moreover, removal of epithelium did not affect ATP-elicited contraction, whereas ATP-induced relaxation was significantly augmented. These data suggest that P_{2X}- and P_{2Y}- purinoceptors are localized in smooth muscle cells and that the relaxant response is probably modulated by excitatory factor(s) released by epithelial cells.

Keywords: ATP; rat urinary bladder; P_2-purinoceptors

Introduction

The actions of purines as neurotransmitters/neuromodulators have been studied in various smooth muscle preparations (Kennedy, 1990). These actions are mediated via receptors which have been classified as P_1-and P_2-purinoceptors, preferentially recognizing adenosine and adenosine-5'-triphosphate (ATP), respectively (Burnstock, 1978). Largely on the basis of the different structure-activity relationships of ATP analogues, P_2-purinoceptors on smooth muscle have been subdivided into P_{2X}- and P_{2Y}-purinoceptors, generally associated with contraction and relaxation, respectively (Burnstock & Kennedy, 1985). However, it has been proposed recently that the agonist-potency order cannot be taken as an absolute parameter for P_2-purinoceptor classification, but that information on transduction mechanisms and molecular structure has also to be taken into account. This has led to the proposal of the existence of two P_2-purinoceptor families: the ionotropic P_{2X}-purinoceptor family, mediating fast and transient responses to ATP, and the metabotropic P_{2Y}-purinoceptor family, mediating slower responses through G-proteins (Abbracchio & Burnstock, 1994; Barnard *et al.*, 1994; Fredholm *et al.*, 1994).

Among its many biological effects, ATP is known to affect bladder function. Non-cholinergic, non-adrenergic, nerve-mediated contraction of the detrusor involving ATP as neurotransmitter has been known for many years (Langley & Anderson, 1985), whereas few studies suggest a possible role for ATP in the non-cholinergic, non-adrenergic, nerve-mediated relaxation of the detrusor (Klarskov, 1987a,b). In particular, in mouse carbachol-prestimulated bladder, ATP induced a biphasic force response: a marked contraction followed by a sustained relaxation (Boland *et al.*, 1993). The nature of the P_2-purinoceptor(s) mediating these functional effects still needs to be fully characterized.

The aim of the present study was to investigate the response(s) induced by ATP (and ATP analogues) in rat bladder at resting tone and in the carbachol prestimulated bladder strips, in order to confirm a possible role of ATP in nerve-mediate detrusor relaxation and tentatively define the purinoceptor subtype(s) involved in this response and their localization.

Methods

Muscle preparation

Male Sprague-Dawley rats weighing 175–200 g were used for all experiments. The animals were killed by cervical disloca-

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tion. The abdomen was opened and the entire urinary bladder cut below the trigone, rapidly removed and opened along its longitudinal axis. The organ was placed in a 10 ml or 20 ml organ bath, perfused (rate: 60 ml h^{-1}) with Krebs solution at 37°C continuously bubbled with a 95% O_2 and 5% CO_2 mixture. The Krebs solution had the following composition (mM): NaCl 113, KCl 4.7, $CaCl_2$-$6H_2O$ 2.5, KH_2PO_4 1.2, $NaHCO_3$ 25, $MgSO_4$-$7H_2O$ 1.2, glucose 11.5, pH 7.4.

A resting load of 2 g (19.6 milliNewton, mN) was applied, and the preparation was allowed to equilibrate for 60 min before the start of the experiment. The effects of ATP and related compounds were examined both in the resting bladder and in the bladder prestimulated with carbachol. When studying the role of epithelial cells in the action of the drugs under investigation, the epithelium was removed by treatment with a 0.15% (w/v) collagenase Krebs solution for 3 min as previously described (Pinna *et al.*, 1992). The integrity of smooth muscle was evaluated with 1 μM acetylcholine.

Force was recorded by means of an isometric microdynamometer consisting of a strain gauge transducer, a high-gain amplifier and a galvanometer (2-channel recorder Gemini, 7070, Basile, Italy). Concentration-response curves to ATP and related compounds were obtained by a cumulative increase in the total concentration of agonist tested in 1 μM carbachol-precontracted tissues. Because of its desensitizing effect, the concentration-response curves to α,β-methyleneATP (α,β-meATP) were not cumulative. In this case suramin was added in perfusion. After the pharmacological evaluation, the tissue was carefully dessicated by means of a vacuum-dessicator and the dry weight determined. Each response was expressed either as mN force per mg dry weight (d.wt.) or as % of control response.

Materials

Acetylcholine chloride (ACh), adenosine (ADO), adenosine-5'-triphosphate (ATP), α,β-methyleneATP (α,β-meATP), carbachol, guanosine 5'-O-(2-thio-diphosphate) (GDPβS), guanosine 5'-O-(3-thiotriphosphate) (GTPγS), 2-methylthioATP (2-meSATP), 5'-N-ethyl-carboxamidoadenosine (NECA), nitrobenzyl-thioinosine (NBTI), 8-phenyl-theophylline (8-PT) and uridine-5'-triphosphate (UTP) were from Sigma. Suramin was a kind gift from Bayer. All drugs were dissolved in Krebs solution (except for 8-PT which was dissolved in 80% methanol containing 0.2 M NaOH) and added to the smooth muscle bath in volumes of 0.15 ml or less. All other chemicals were reagent grade and were obtained from Sigma.

Statistical analysis

The data in the test are expressed as mean ± standard error of 4–36 experiments. Statistical analysis of the data was performed with Student's *t* test. A probability value of $P<0.05$ was considered statistically significant.

Results

Figure 1a shows the effects of ATP on rat urinary bladder resting tone, At 1 mM concentration, ATP exerted a clear biphasic effect, consisting of a rapid and transient contraction (0.76 ± 0.06 mN mg^{-1} d.wt., $n=36$), followed by a slower sustained relaxation (0.10 ± 0.02 mN mg^{-1} d.wt., $n=36$, Figure 1a(i)). After washing the tissue twice, the second application of 1 mM ATP, 10 min after the first challenge, was characterized by a marked reduction of contraction (residual response was 43 ± 3% of initial response, $P<0.01$), whereas relaxation did not seem to be affected (Figure 1a(ii)). A complete restoration of the biphasic response to ATP could be obtained after 1 h (Figure 1a(iii)). To demonstrate the relaxation phase more clearly, a concentration-response curve was performed on the carbachol (1 μM) prestimulated bladder (Figure 1b). Under these experimental conditions the relaxant response was also detectable at 30 μM ATP (0.04 ± 0.007 mN mg^{-1} d.wt., $n=15$) and was maximal at 1 mM (0.5 ± 0.05 mN mg^{-1} d.wt., $n=15$). No further relaxation was obtained with 3 mM ATP (data not shown).

The possible desensitizing effect of the P_{2X}-purinoceptor agonist, α,β-methyleneATP (α,β-meATP) was tested in experiments shown in Figure 2. Application of the selective P_{2X}-agonist at 5 μM concentration resulted in a marked reduction

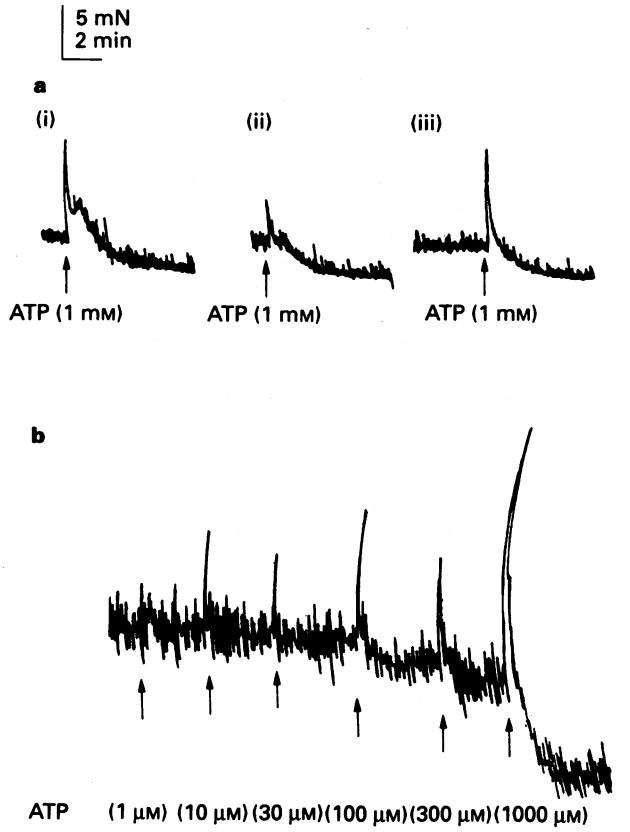

Figure 1 ATP induces a biphasic response in rat urinary bladder. (a) Original tracing showing contraction and relaxation induced by ATP in rat urinary bladder preparation at resting tone. The intervals between the first (i) and the second (ii) challenge and between the second and the third (iii) challenge with agonist were 10 and 60 min, respectively. (b) Original tracing showing the contractile and relaxant responses to ATP in tissue prestimulated with 1 μM carbachol.

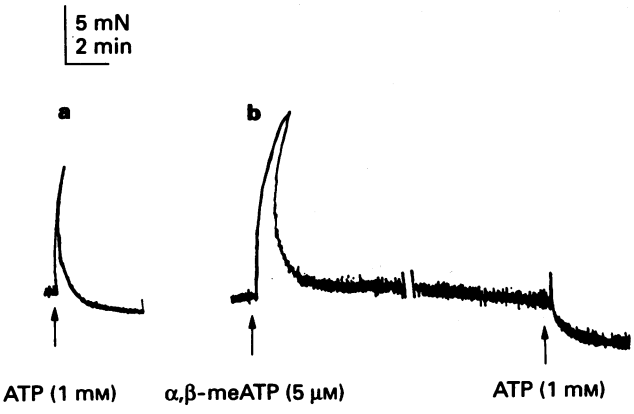

Figure 2 Representative tracing showing desensitization induced by α,β-methylene ATP (α,β-meATP) on the ATP contractile response of rat urinary bladder. The interval between the first ATP challenge (a) and the α,β-meATP treatment (b) was 60 min. The α,β-meATP contact time was 20 min. The second ATP challenge was performed after a further 30 min.

of the tissue contraction to ATP (compare contraction in Figure 2b with Figure 2a), without affecting the relaxant response. Interestingly and consistent with its selectivity on P_{2X}-receptors, α,β-meATP produced only a sustained monophasic contractile response (1.41 ± 0.08 mN mg^{-1} d.wt., n = 21) which was significantly higher than the ATP-induced contraction ($P<0.001$).

Conversely as shown in Figure 3a, the P_{2Y}-purinoceptor agonist, 2-methyl-thio-ATP (2-meSATP), induced a concentration-related relaxation on carbachol prestimulated strips. Consistent with its apparent selectivity for P_{2Y}-purinoceptors, a high concentration (1 mM) of this agonist mediated a significantly greater relaxation with respect to ATP (0.50 ± 0.05 vs 0.89 ± 0.014 mN mg^{-1} d.wt., $n = 4$). Since it has been reported that P_{2Y}-purinoceptors are linked to G-protein activation (Kennedy, 1990; Abbracchio & Burnstock, 1994), we have assessed whether G-proteins are involved in any of the ATP-induced effects by utilizing G-protein modulators, such as guanosine 5'-O-(3-thiotriphosphate) (GTPγS) (which is known to permanently activate G-proteins) and 5'-O-(2-thio-diphosphate) (GDPβS) (which conversely stabilizes G-proteins in their inactive state). Results are shown in Figure 3b. GTPγS (10 μM) amplified ATP-induced relaxation without affecting the contractile phase ($n = 3$, Figure 3b(i)). Conversely, 100 μM GDPβS completely inhibited only ATP-induced relaxation (Figure 3b(ii)), further confirming that G-protein coupling is involved only in the later ATP-dependent relaxing phase.

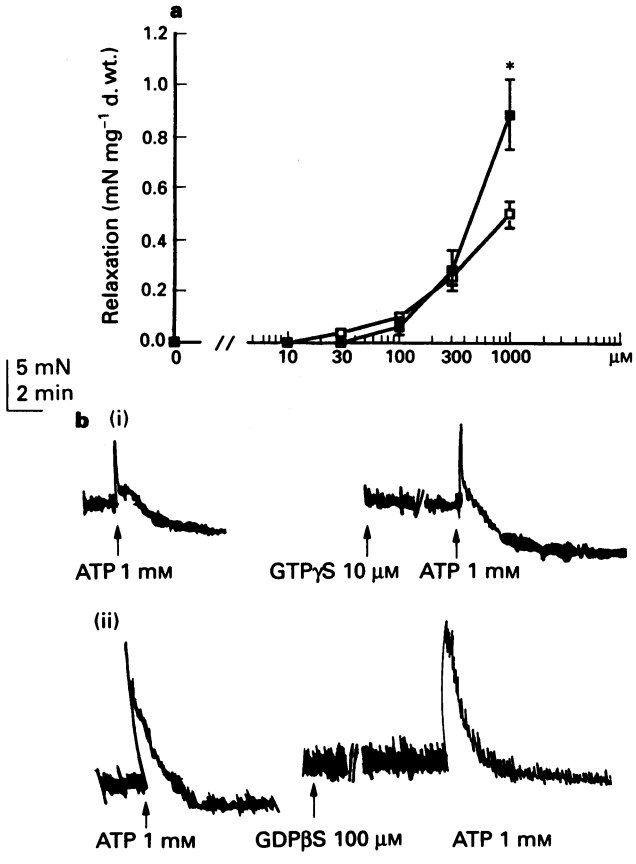

Figure 3 Involvement of P_{2Y}-purinoceptors in ATP-induced relaxation of rat bladder. (a) Concentration-response curves for relaxation to ATP (□) and 2-methyl-thioATP (2-meSATP) (■) in rat urinary bladder strips precontracted with 1 μM carbachol. Values represent the mean ± s.e.mean of 4 separate experiments. (b) Representative trace showing the effect of guanosine 5'-O-(3-thiotriphosphate) (GTPγS) (i) and guanosine 5'-O-(2-thio-diphosphate) (GDPβS) (ii) on ATP-induced responses. Rat urinary bladder preparations at resting tone were exposed to either GTPγS or GDPβS for 20 min before application of the second ATP challenge.

Since ATP is known to be quickly hydrolyzed to adenosine (Cusack & Hourani, 1984), in order to investigate the possible contribution of this metabolite to the detected responses (and to ATP-induced relaxation in particular), we have performed experiments in the presence of modulators of adenosine effects, such as the adenosine uptake inhibitor, nitrobenzyl-thioinosine (NBTI) and the adenosine receptor antagonist, 8-phenyltheophylline (8-PT). As shown in Figure 4a(i), NBTI did not significantly affect either the basal resting tone or ATP-induced responses. In the presence of NBTI, the ATP-induced relaxation was not significantly different from that obtained in the absence of the uptake inhibitor, apparently ruling out any significant contribution of adenosine to the detected response. Conversely, NBTI significantly potentiated the relaxation induced by exogenously added adenosine (Figure 4a(ii)). In fact, in NBTI-pretreated bladder strips, adenosine-induced relaxation was 250 ± 20% of the relaxation measure in the absence of the uptake inhibitor ($P<0.001$). In the presence of 8-PT, the relaxant responses evoked by ATP were not significantly modified (Figure 4b). That ATP and adenosine induce independent relaxation is confirmed by data shown in Figure 5a: ATP-induced relaxation was additive with the relaxation induced by the hydrolysis-resistant adenosine analogue, 5'-N-ethylcarboxamido-

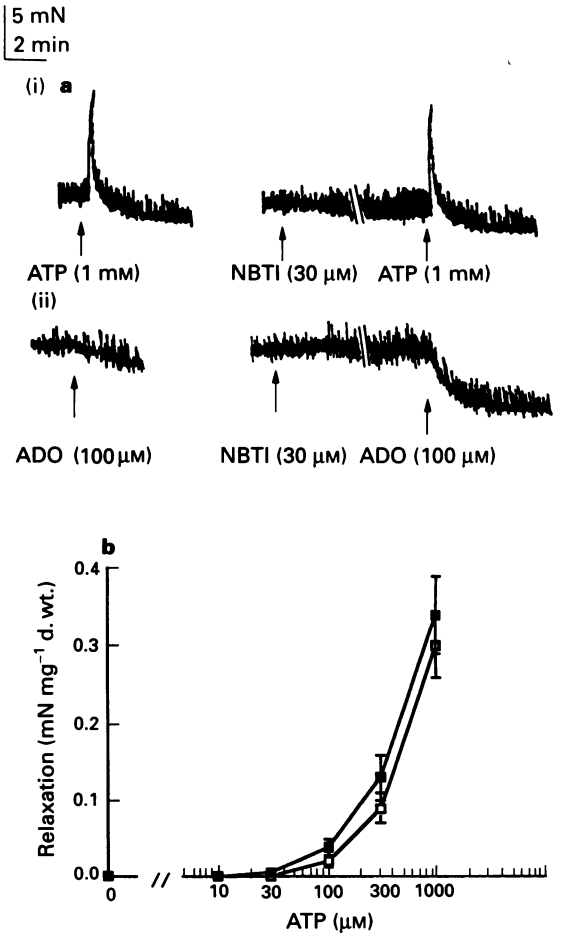

Figure 4 Adenosine does not contribute to the effects induced by ATP in rat urinary bladder. (a) Typical tracing showing the effect of nitro-benzyl-thioinosine (NBTI) on responses to ATP (i) and adenosine (ADO) (ii) in bladder preparations at resting tone. NBTI was added 1 h after the initial challenge with agonists. The second challenge with agonists was performed 15 min afterwards. (b) Concentration-dependent relaxation of rat urinary bladder induced by ATP in absence (□) or presence (■) of 100 μM 8-phenyltheophylline (8-PT). Relaxation was evaluated in preparations precontracted with 1 μM carbachol. 8-PT was added 20 min before starting the second concentration-response curve. Values are the mean ± s.e.mean of 8 separate experiments.

adenosine (NECA), suggesting the involvement of different receptors. In these experiments, NECA was tested at 10 μM, which induced a maximal relaxation as illustrated in Figure 5b.

We also tested the effects of the P_2-purinoceptor antagonist, suramin. Suramin (50 μM) did not modify significantly either ATP-induced contraction or ATP-induced relaxation ($n = 4$, Figure 6a and 6b). Notably, α,β-meATP concentration-response curve was shifted to the right by suramin in the same system ($n = 4$, Figure 7).

Figure 8 shows the effect of uridine-5'-triphosphate (UTP) on resting tone. No effect was detected at concentrations lower than 1 mM, at which UTP induced a potent monophasic contraction (0.67 ± 0.09 mN mg^{-1} d.wt., $n = 6$, Figure 8a), which was not statistically different from the contraction induced by ATP. Unlike the contractile response to ATP (Figure 2), the contractile response to UTP was not desensitized by pretreatment with α,β-meATP (Figure 8b).

In order to clarify the localization of the two different purinoceptor subtypes involved in the ATP-mediated responses, we evaluated the response to ATP in urinary bladder strips devoid of epithelium. The relaxant response evoked by 1 mM ATP at resting tone was significantly enhanced (0.10 ± 0.02 vs 0.29 ± 0.03 mN mg^{-1} d.wt., $n = 36$), whereas the contractile response was unaffected (0.76 ± 0.06 vs 0.76 ± 0.05 mN mg^{-1} d.wt., $n = 36$) by removal of epithelium. The integrity of the smooth muscle was evaluated with 1 μM ACh: no difference in response to ACh was detected in the presence or absence of epithelium (0.92 ± 0.08 vs 1.1 ± 0.08 mN mg^{-1} d.wt.).

Discussion

The existence of a purinergic innervation in bladder was initially proposed by Brown *et al.* (1979), who demonstrated that high concentrations of ATP caused contraction and that adenosine produced relaxation of the rat urinary bladder. Subsequent studies have confirmed the contractile action of ATP in guinea-pig (Cusack *et al.*, 1987; Hoyle *et al.*, 1990), in rabbit (Creed *et al.*, 1991) and in rat (Bo & Burnstock., 1992; Nicholls *et al.*, 1990) urinary bladder.

The pharmacological characterization performed in these studies suggested that the contraction evoked by ATP was mediated by the P_{2X} subtype of the P_2-purinoceptor (Burn-

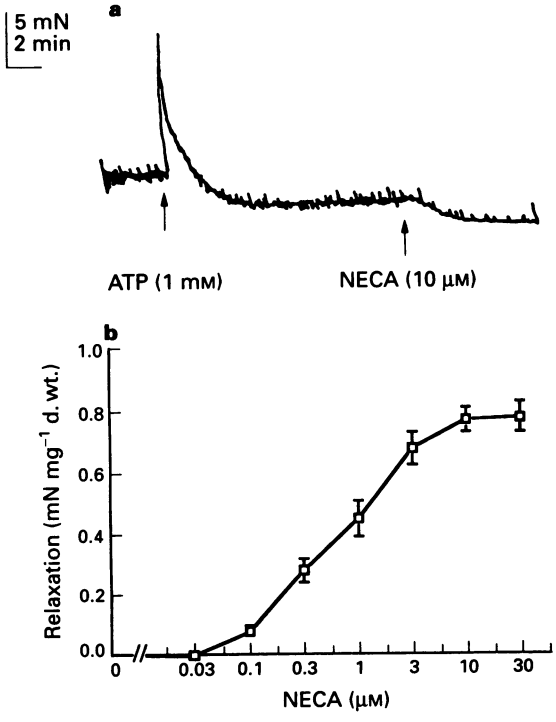

Figure 5 5'-N-ethylcarboxamidoadenosine (NECA) and ATP induce independent relaxation of rat bladder. (a) Effect of NECA on ATP-induced relaxation in rat urinary bladder at resting tone. (b) Concentration-response curve to NECA. Relaxation was assayed in preparations precontracted with 1 μM carbachol. Values represent the mean ± s.e.mean of 10 experiments.

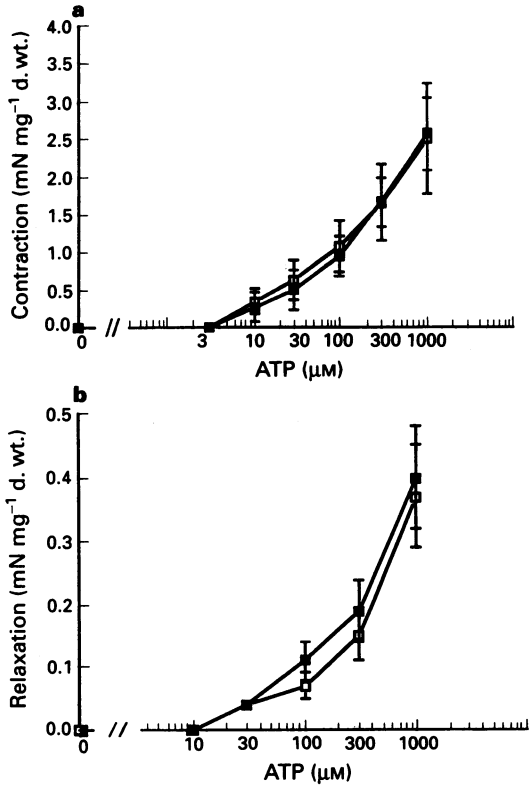

Figure 6 Effect of suramin on ATP-induced responses in rat urinary bladder. (a) ATP-induced contraction in the absence (□) or presence (■) or 50 μM suramin. (b) ATP-induced relaxation in the absence (□) or presence (■) of 50 μM suramin. Relaxation was assayed on rat bladder strips precontracted with 1 μM carbachol. Rat bladder preparations were exposed to suramin for 90 min before starting the second concentration-response curve for ATP. Values are the mean ± s.e.mean of 4 experiments.

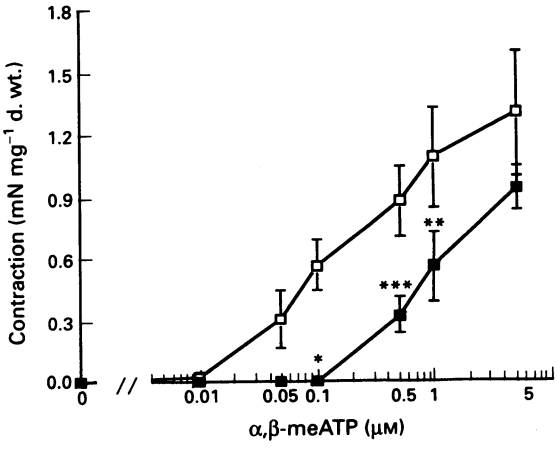

Figure 7 Effect of suramin on α,β-methylene ATP (α,β-meATP)-induced responses in rat urinary bladder. Concentration-response curves for α,β-meATP in the absence (□) and presence (■) of 50 μM suramin. The rat urinary bladder strips were precontracted with carbachol (1 μM) and suramin was added by perfusion. Values are the mean ± s.e.mean of 4 experiments.

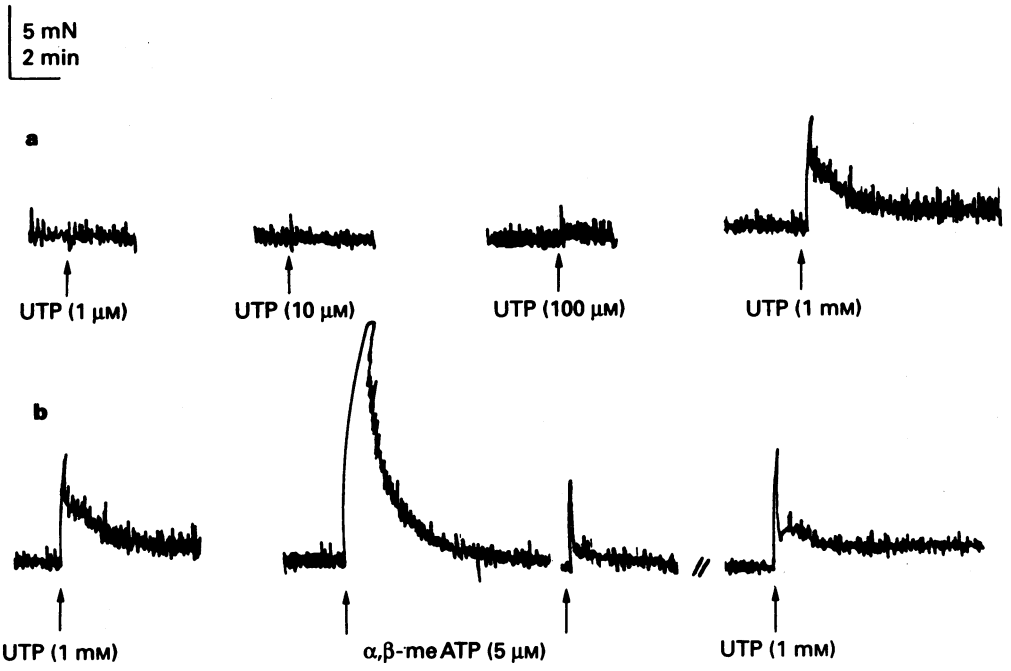

Figure 8 Typical tracings showing UTP-induced responses on urinary bladder strips at resting tone. (a) Concentration-related responses to UTP. (b) Effect of desensitizing α,β-methyleneATP (α,β-me ATP) concentrations on UTP-induced contraction. The first challenge with α,β-meATP was performed 1 h after the UTP application. After 30 min the tissue was washed and a second application of α,β-meATP was made, followed by the second UTP challenge.

stock & Kennedy, 1985). Dahlen & Hedqvist (1980) also reported an inhibitory action of ATP on transmural nerve stimulated contraction of rat urinary bladder preparations, implying the existence of multiple ATP receptors in this tissue. Furthermore, non-adrenergic, non-cholinergic nerve-mediated relaxation was discovered in pig and human detrusor (Klarskov, 1987a). More recently, Boland *et al.* (1993) have demonstrated for the first time that, besides its contractile action, ATP could also relax mouse urinary smooth muscle in carbachol-stimulated preparations, suggesting the involvement of P_{2Y}-purinoceptors.

In our study, we have investigated the action of ATP on resting tone and in carbachol precontracted strips in rat urinary bladder. The results show that ATP induces a biphasic response, characterized by an initial fast and transient contraction and by a subsequent slower long-lasting relaxation. To our knowledge, this is the first time that ATP (albeit at high concentration) has been reported to induce a direct relaxation of rat urinary bladder. Furthermore, in carbachol-prestimulated tissues, a concentration-dependent relaxation has been obtained. Under these experimental conditions, a relaxant response was detectable at 30 μM ATP and was maximal at 1 mM.

Formation of adenosine from ATP has been reported in guinea-pig urinary bladder preparations (Cusack & Hourani, 1984). In particular, 5 min after ATP addition, adenosine amounted to 20% of the active compounds in the incubation medium. With rabbit urinary bladder, on the other hand, no significant decrease in the ATP concentration present in organ bath was detected after 48 min (Levin *et al.*, 1981). Nicholls *et al.* (1992) demonstrated that in the rat urinary bladder preparations, about 25% of the ATP was degraded after 5 min but little adenosine was formed.

To rule out the possibility that adenosine could in any way contribute to the detected responses, we have performed experiments with modulators of adenosine effects. Results show that ATP-induced responses were not affected by either NBTI or 8-PT, ruling out the involvement of adenosine; this conclusion was also confirmed by the observation that ATP and NECA-induced relaxant effects were additive.

Experiments performed with the P_{2X}-selective agonist, α,β-meATP confirmed that the contractile phase could indeed be due to the activation of P_{2X}-purinoceptors.

The concentration-dependent relaxant responses obtained with the P_{2Y} agonist, 2-meSATP, suggest that ATP could indeed activate P_{2Y}-like receptors in rat urinary bladder. However, recent studies suggest that responses to this analogue cannot be taken as unequivocal proof for P_{2Y}-purinoceptor involvement. 2-meSATP can undergo enzymatic degradation *in vitro* similarly to ATP. In recent studies (Evans & Kennedy, 1994), in which ecto-ATPase activity was absent or blocked, 2-meSATP showed equivalent potency to α,β-meATP at P_{2X}-purinoceptors, suggesting caution in the interpretation of results obtained with this analogue.

However, the experiments performed with GTPγS and GDPβS, demonstrating amplification and inhibition, respectively, of ATP-induced relaxation, allow us to conclude that the purinoceptor mediating bladder relaxation belongs to the P_{2Y}-purinoceptor family (Abbracchio & Burnstock, 1994). On the other hand, these experiments also indicated that receptors involved in the initial transient contraction do not belong to the G-protein-linked purinoceptor superfamily, confirming that this response may be subserved by a receptor-regulated ion channel (Bean, 1990; Abbracchio & Burnstock, 1994).

Experiments with the P_2-purinoceptor antagonist, suramin, demonstrated that, unlike results reported for P_{2X}-purinoceptors in other tissues (Burnstock, 1993) and other species (Hoyle *et al.*, 1990), ATP-evoked responses were not affected by suramin, which is consistent with previous observations in mouse vas deferens (Von Kugelgen *et al.*, 1990). However, at least for the contractile effects induced by ATP, we were able to demonstrate that suramin can indeed cause a rightward shift of the concentration-response curve to the hydrolysis-resistant ATP analogue, α,β-meATP. This apparent discrepancy can now be interpreted in the light of the recently reported ability of suramin to block ecto-ATPase in the same concentration-range over which it exerts P_{2X}-purinoceptor antagonism (Crack *et al.*, 1994). The absence of any effect on the ATP concentration-response curve may therefore be attri-

buted to the ability of suramin to inhibit the degradation of ATP, consequently opposing the rightward shift of the curve, rather than to suramin-insensitivity of P_{2X}-purinoceptors. On the basis of the results, we can conclude that the P_{2X}-purinoceptor-mediating contraction of rat bladder is indeed suramin-sensitive.

Suramin did not significantly affect ATP-evoked relaxation. Whether this is due to a real receptor insensitivity to suramin or to effects similar to those above described for P_{2X}-purinoceptors still remains to be clarified. The utilization of new hydrolysis-resistant ATP derivatives endowed with P_{2Y}-purinoceptor selectivity (Abbracchio & Burnstock, 1994) will help in elucidating this point.

Since it has been reported that purinoceptors involved in the ATP responses could also respond to UTP (Abbracchio & Burnstock, 1994), we have also tested the action of UTP in our preparations. UTP induced only a rapid and concentration-dependent contraction of rat bladder preparations, which was not desensitized by α,β-meATP, suggesting that ATP and UTP might utilize different receptors. Although further experiments are needed before any conclusion can be drawn on this particular aspect, recent evidence suggests that an ATP/UTP receptor with similar characteristics is also present in the pulmonary vasculature (Rubino & Burnstock, 1994).

The experiments carried out in tissues devoid of epithelial cells indicated a possible localization in smooth muscle for both the P_{2X} and P_{2Y}-purinoceptors. In fact, the contractile phase was not modified by removal of the epithelium, whereas the relaxant phase was significantly increased. The latter result also suggested a modulation of relaxation by excitatory factor(s) released from the epithelium.

In conclusion, in rat urinary bladder, ATP coreleased with acetylcholine activates two subtypes of purinoceptors mediating different and opposite functional effects. In particular, the inhibitory action evoked by P_{2Y}-purinoceptors might counteract P_{2X}-purinoceptor-mediated contraction, therefore suggesting that ATP might play an important functional role in the physiological regulation of both micturition and bladder filling. This would also suggest a role for P_{2Y}-purinoceptors in the high compliance of urinary bladder and will be important in future studies aimed at assessing a possible defect of purinoceptors in pathological conditions characterized by bladder malfunctions.

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