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ATP induced-relaxation in the mouse bladder smooth muscle

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1 The effect of adenosine 5'-triphosphate (ATP) on the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) as measured with the fluorescent Ca^{2+}-indicator fura-2, and on force was investigated in the intact smooth muscle strips of the mouse urinary bladder.

2 ATP elicited, when exogenously applied, a large increase of $[Ca^{2+}]_i$ with limited force development resulting in a marked Ca^{2+}-force dissociation.

3 Release of endogenous neurotransmitters by transmural electrical stimulation (TES) for 30 s induced a steady increase of $[Ca^{2+}]_i$ and a peak contraction, followed within 15 s by a relaxation.

4 In carbachol-prestimulated preparations, ATP elicited an initial rise of $[Ca^{2+}]_i$ followed by a return to the initial precontraction Ca^{2+}-level. Force in contrast presented a biphasic pattern, i.e. an initial contraction was followed by a sustained relaxation.

5 In the K^+-depolarized precontracted preparation, ATP elicited a slight initial rise of $[Ca^{2+}]_i$. The partial relaxation of the force during depolarization was not preceded by a transient contraction.

6 The ATP-induced relaxation of the K^+-prestimulated preparations was not inhibited by 8-phenyltheophylline, a potent P_1-purinoceptor antagonist.

7 The order of potency for relaxation of the ATP analogues was 2-MeSATP > ATP > βγMe-ATP, which is characteristic for P_{2y}-purinoceptors.

8 These results indicate that, besides its activating effect, ATP also relaxes the mouse urinary bladder. It is suggested that the relaxant effect, mediated through P_{2y}-purinoceptors, is mainly responsible for the low contractile potency of ATP in the bladder.

Keywords: ATP; P_2-purinoceptors; cytosolic Ca^{2+}; urinary bladder

Introduction

Receptors for extracellular purines have been described in numerous tissues (Gordon, 1986). The purinoceptors for adenosine 5'-triphosphate (ATP) in the smooth muscle tissues were subclassified into contracting P_{2x}- and relaxing P_{2y}-purinoceptors (Burnstock & Kennedy, 1985). The neurotransmitter function of ATP was initially reported in the smooth muscle of the rodent vas deferens in which co-transmission of ATP and noradrenaline occurs (Sneddon & Westfall, 1984). In this tissue, ATP elicits contraction through a Ca^{2+} influx induced by P_{2x}-purinoceptor activation (Friel, 1988). Co-transmission of ATP and acetylcholine was described in the smooth muscle of the rodent urinary bladder (Burnstock *et al.*, 1978), in which ATP also induces contraction through the P_{2x}-purinoceptors by Ca^{2+} influx (Acevedo & Contreras, 1989; Katsuragi *et al.*, 1990).

However, the contractile potency of ATP is very low both in the vas deferens (Fedan *et al.*, 1982) and in the bladder (Acevedo & Contreras, 1989; Hoyle & Burnstock, 1989; Katsuragi *et al.*, 1990). We observed recently that the low contractile effect of ATP in the mouse vas deferens occurs in spite of a marked increase of the free cytosolic calcium concentration ($[Ca^{2+}]_i$) and proposed that it mainly resulted from the binding of ATP to both P_{2x}- and P_{2y}-purinoceptors (Boland *et al.*, 1992). The present experiments were carried out to test the hypothesis that the low contractile potency of ATP observed in the bladder smooth muscle is due to the activation of both contracting and relaxing purinoceptors. We therefore measured the effect on $[Ca^{2+}]_i$ and force induced by ATP in resting and in precontracted bladder preparations.

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Methods

Muscle preparation

Adult male albino mice (NMRI, 3–4 month old, 30–40 g) were killed by cervical dislocation after anaesthesia with ether. The urinary bladder was isolated and transferred to the oxygenated HEPES-buffered Krebs solution at room temperature. The bladder was dissected free from its surrounding tissues and opened along its medial axis to allow removal of the thick mucosa by gentle rubbing. Intact smooth muscle strips (10 × 4 mm) were dissected.

Measurements of cytosolic Ca^{2+} and of force

The bladder strips were loaded for 3 h with 5 μM fura-2AM, as previously described (Katsuragi *et al.*, 1990). This loading procedure did not affect either the amplitude or the time-course of the force response to 10 μM carbachol ($n = 4$). After the loading, the strips were rinsed in the HEPES-Krebs solution for 30 min. The mounting of intact smooth muscle preparations under isometric conditions and the experimental set-up used for the simultaneous measurements of fura-2 fluorescence and force response have been described (Himpens & Somlyo, 1988). The bladder strips were stretched to a passive tension of 5 mN. The 340/380 nm fura-2 ratio was continuously recorded during the experiments. An internal calibration of the fura-2 fluorescence signals into free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was performed at the end of each experiment by use of the procedure designed by Himpens *et al.* (1988). The increases of $[Ca^{2+}]_i$ and of force induced by the stimulation with 10 μM carbachol were used as references. The changes of $[Ca^{2+}]_i$ are expressed in absolute values (nM) or as a percentage of the increase of $[Ca^{2+}]_i$ in 10 μM carbachol (100%). The force response is expressed in absolute values (mN) or as a percentage of the maximal force increase obtained with 10 μM carbachol (100%).

Stimulating procedure

The bladder strips were continuously superfused at 4 ml min^{-1}. Contraction was obtained by superfusing the preparation with 10 μM carbachol or by 70 mM K^+. The effect of ATP was examined both in the resting bladder and in the bladder prestimulated by carbachol or by 70 mM K^+. Transmural electrical stimulation (TES) specific for the nerve endings was applied as 30 s train of 1 ms square pulses of supramaximal voltage at 25 Hz through platinum electrodes lying in parallel to the smooth muscle preparation. The specificity of this TES for the nerve endings was indicated by the total abolition of the contractile response by 1 μM tetrodotoxin, as previously reported for similar pulse parameters (Parija et al., 1991).

Solutions

The normal HEPES-Krebs solution contained in mM: NaCl 135.5, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.5, HEPES 11.6 and glucose 11.5. The isotonic 70 mM K^+ solution was obtained by replacing external Na^+ by an equivalent amount of K^+. Sodium salt of adenosine triphosphate (ATP), sodium salt of βγ-methylene ATP (βγme-ATP), carbachol and tetrodotoxin were from Sigma. Tetrasodium salt of 2-methylthioadenosine triphosphate (2-MeSATP) was obtained from ICN Biochemicals (Cleaveland, Ohio, U.S.A.). 8-Phenyltheophylline and ionomycin were from Calbiochem. Fura-2AM was from Molecular Probes (Eugene, OR, USA). All ATP analogues were the D-isomers. Drugs were dissolved in the HEPES-Krebs solution, except for 8PT which was dissolved in 80% methanol containing 0.2 M NaOH (Griffith et al., 1981). All other reagents were of analytical grade.

Statistics

The results are presented as means ± standard error of mean (s.e.mean), and n is the number of experiments. The data were evaluated for differences by Student's t test (paired two-tailed t test). A probability of less than 0.05 was considered significant.

Results

The mouse bladder smooth muscle did not show spontaneous contractile activity in the normal Krebs solution containing 1.5 mM Ca^{2+} at room temperature ($n = 10$), as previously reported (Acevedo & Contreras, 1989). During these resting conditions, the values of the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and of the force were 109 ± 4 nM and 5 ± 0.8 mN respectively. These levels were used as the basal references (0%) as mentioned in the methods. Our value of resting $[Ca^{2+}]_i$ in the mouse bladder muscle tissue (109 nM) is similar to that reported in the isolated smooth muscle cells of guinea-pig bladder (Ganitkevich & Isenberg, 1991). The effect of superfusion for 2 min with 10 μM carbachol, 70 mM K^+ and 100 μM ATP are compared in Figure 1. Carbachol (10 μM) increased the values of $[Ca^{2+}]_i$ to 242 ± 27 nM and of force to 25 ± 2 mN ($n = 9$). To these values, all the measurements described below were normalized (100%). During depolarization with 70 mM K^+, $[Ca^{2+}]_i$ and force attained a level of 351 ± 60 nM (181%) and of 23 ± 3 mN (89%) respectively ($n = 7$). In contrast, the rise of $[Ca^{2+}]_i$ elicited by 100 μM ATP was 265 ± 30 nM (117%) while force increased to only 6 ± 1 mN (2%) ($n = 6$). Two other preparations even failed to contract in response to ATP application, as reported in the human bladder (Hoyle et al., 1989), in spite of a marked elevation of $[Ca^{2+}]_i$. Thus, in spite of a large increase of $[Ca^{2+}]_i$, superfusion with exogenous ATP elicited only a very weak contraction.

In order to analyse the effects of endogenous ATP, we induced the release of neurotransmitters from the bladder by

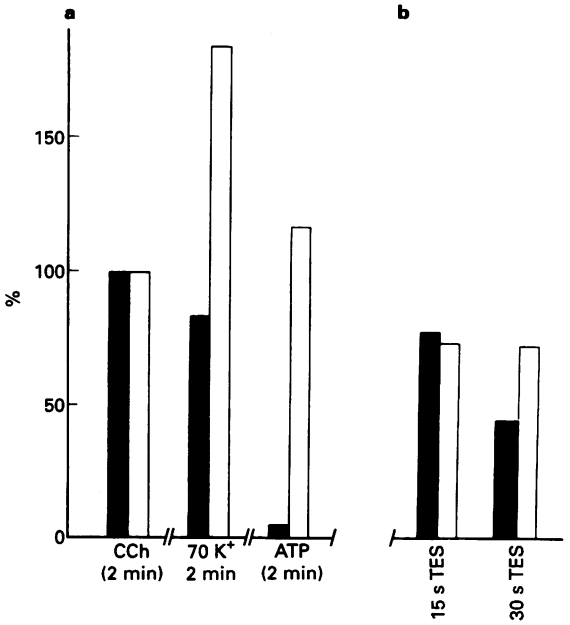

Figure 1 (a) Peak increases of $[Ca^{2+}]_i$ (open columns) and of force (solid columns) above the resting level induced by 70 mM K^+ and by 100 μM ATP, as normalized to the values elicited by 10 μM carbachol (CCh). (b) Comparison of the increases of $[Ca^{2+}]_i$ (open column) and of force (solid column) after 15 and 30 s of transmural electrical stimulation (TES).

transmural electrical stimulation (TES). TES with pulses of short duration is specific for the nerve endings, and does not activate the smooth muscle cells. The TES-induced increases of $[Ca^{2+}]_i$ and force for 15 and 30 s stimulation are displayed in Figure 1b while an example is presented in Figure 2. TES for 30 s with 1 ms square pulses of 40 V at 25 Hz induced within 15 s the peak of $[Ca^{2+}]_i$ to 207 ± 20 nM (73%) and of force to 20 ± 0.8 mN (76%) ($n = 8$) (Figure 2). Thereafter, during continued stimulation, $[Ca^{2+}]_i$ remained at about 200 nM while force declined by 40 ± 6%. At the end of the TES, $[Ca^{2+}]_i$ returned within 1 min to its basal level while the resting force level was reached in about 20 s. Incubation of the bladder preparation for 10 min with 1 μM tetrodotoxin completely abolished the response to TES indicating that this procedure elicited no direct stimulation of the smooth muscle cells ($n = 4$). The TES-induced contraction was reduced by 65 ± 7% by preincubation for 5 min with 1 μM atropine. In this condition, the force also started to decline after 15 s stimulation in spite of a maintained Ca^{2+} signal ($n = 3$).

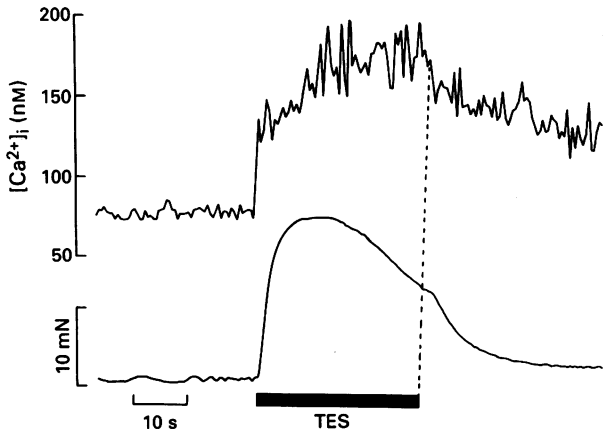

Figure 2 Record showing the changes in $[Ca^{2+}]_i$ (upper trace) and of force (lower trace) elicited during transmural electrical stimulation (TES) with 1 ms square pulses of 40 V at 25 Hz for 30 s. The dashed line represents the end of the electrical stimulation.

The force-Ca^{2+} dissociation observed during stimulation with both exogenous and endogenous ATP suggested the possibility of a relaxing effect by ATP. In order to investigate this possibility, we performed stimulations by ATP in bladder preparations precontracted by carbachol or by K^+-depolarization. A steady raised-tone was first obtained by stimulation with 10 μM carbachol. After the initial peak contraction (100%, see above) force stabilized within 20 min to a level of 20 ± 2% ($n = 8$). Preliminary data showed that application of 100 μM ATP for 5 min to this prestimulated preparation induced a contraction to 32% ($n = 6$), followed after 5 min by a return of the force to the previous raised-tone level (20%). However, during prolonged stimulation with 100 μM ATP for more than 10 min we found a force relaxation below the raised-tone level, which stabilized at 14 ± 2% ($n = 4$). We further examined the effect on $[Ca^{2+}]_i$ and force of 1000 μM ATP in the carbachol-precontracted bladder. First, $[Ca^{2+}]_i$ increased from 157 ± 20 to 275 ± 50 nM and force rose from 20 ± 2 to 29 ± 3% ($n = 5$) (Figure 3). Then, within 5 min, both $[Ca^{2+}]_i$ and force returned to their previous levels. Finally, after 10 min superfusion with 1000 μM ATP, $[Ca^{2+}]_i$ remained at 164 ± 28 nM while force relaxed to 7 ± 1%. On washing out ATP, both $[Ca^{2+}]_i$ and force returned to their previous carbachol-induced level. A second stimulation with 1000 μM ATP at 10 min interval elicited within 2 min slight rises of $[Ca^{2+}]_i$ to 202 ± 34 nM and of force to 26 ± 4% ($n = 5$) (Figure 3). Thereafter, during the prolonged ATP stimulation, $[Ca^{2+}]_i$ returned to 165 ± 21 nM while force relaxed close to the basal resting level (3 ± 1%). Again, on washing out ATP, $[Ca^{2+}]_i$ and force returned to 168 ± 25 nM and 18 ± 2% i.e. back to its control levels.

We also analysed the effects of ATP in the bladder smooth muscle prestimulated with a depolarizing solution containing 1.5 mM Ca^{2+} and 70 mM K^+. After 20 min stimulation with 70 mM K^+, $[Ca^{2+}]_i$ stabilized at 247 ± 35 nM and force at 10 ± 2% ($n = 7$). In this condition, application of 1000 μM ATP slightly elevated $[Ca^{2+}]_i$ within 2 min to 319 ± 69 nM while force hardly increased to 11 ± 3% ($n = 5$) (Figure 4). The ATP-induced increase of $[Ca^{2+}]_i$ was significantly lower in 70 mM K^+ (72 nM) than in 10 μM carbachol (118 nM) ($P < 0.05$). Thereafter, during prolonged stimulation with ATP $[Ca^{2+}]_i$ returned to 250 ± 38 nM and force declined to 5 ± 2%. In the K^+-precontracted bladder, incubation for 30 min with 10 μM 8-phenyltheophylline, a potent antagonist of P_1-purinoceptors, did not modify the relaxation induced by 100 μM ($n = 8$) or 1000 μM ATP ($n = 6$). The rank order of potency for relaxation of ATP analogues (100 μM) was examined in K^+ prestimulated preparations. Under these conditions, steady state force declined by 25 ± 3% after 5 min stimulation with ATP ($n = 3$), by 43 ± 3% after 2-MeSATP incubation and by 9 ± 2% in the presence of βγMe-ATP. The observed rank order of potency for relaxation was thus 2-MeSATP > ATP > βγMe-ATP. Finally, also in guinea-pig ($n = 6$) and rat ($n = 5$) bladder smooth muscles precontracted by 70 mM K^+, stimulation with ATP induced a reproducible, sustained and reversible relaxation (data not shown).

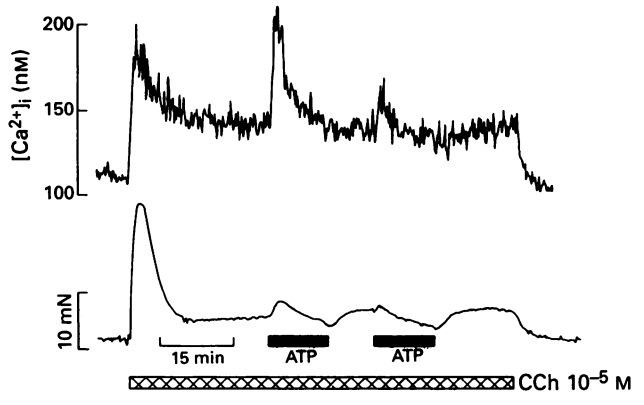

Figure 3 Effects on $[Ca^{2+}]_i$ (upper trace) and on force (lower trace) of two sequential applications of ATP 1000 μM for 10 min in the bladder preparation prestimulated by 10 μM carbachol (CCh).

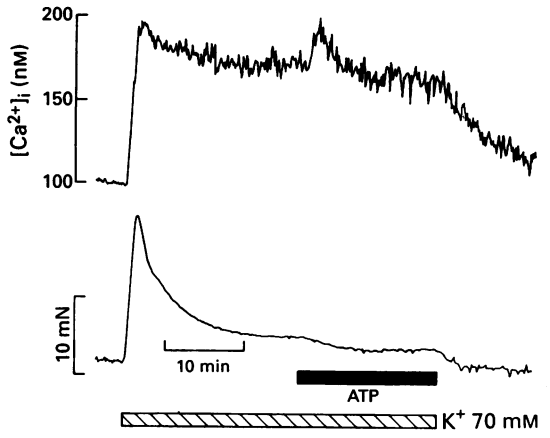

Figure 4 Effects on $[Ca^{2+}]_i$ (upper trace) and force (lower trace) of ATP (1000 μM) in the bladder preparation depolarized by 70 mM K^+. Note that the Ca^{2+} transient evoked by ATP in the K^+-precontracted bladder is smaller than the value in the carbachol-prestimulated bladder.

Discussion

Co-transmission of acetylcholine and ATP was described in the mammalian urinary bladder smooth muscle (Ambache & Aboo Zar, 1970; Burnstock *et al.*, 1972), in which transmural electrical stimulation of the nerve endings induces the activation of postsynaptic muscarinic receptors and purinoceptors (Fujii, 1988; Brading & Mostwin, 1989). In the bladder, the muscarinic receptors induce a release of Ca^{2+} from the internal stores and Ca^{2+} influx through receptor-operated ion channels (Maggi *et al.*, 1989; Iacovou *et al.*, 1990), while the P_{2x}-purinoceptors activate membranous ion permeability resulting in depolarization (Inoue & Brading, 1990) and Ca^{2+} influx (Katsuragi *et al.*, 1990). ATP elicits a large inward current in dispersed bladder smooth muscle cells (Inoue & Brading, 1991).

However, the force response to ATP was reported to be rather limited in the intact urinary bladder smooth muscle from the guinea-pig (Ambache & Aboo Zar, 1970; Burnstock *et al.*, 1978), the rat (Brown *et al.*, 1979; Bhat *et al.*, 1989), the mouse (Acevedo & Contreras, 1989) as well as from human bladder (Hoyle *et al.*, 1989). In these tissues, prolonged application of ATP up to 1000 μM induced only a weak and short contraction. These latter features were usually attributed either to extracellular breakdown of ATP by ectonucleotidases (Brown *et al.*, 1979; Inoue & Brading, 1991), or to the tachyphylaxis of the P_{2x}-purinoceptors (Kennedy, 1990). To investigate further the low potency of ATP, we studied simultaneously for the first time the ATP-induced changes of $[Ca^{2+}]_i$ and of force in intact bladder smooth muscle. The finding that ATP elicited a large increase of $[Ca^{2+}]_i$, which was abolished in the Ca^{2+}-free solution (Acevedo & Contreras, 1989; Bhat *et al.*, 1989; Katsuragi *et al.*, 1990), shows that ATP induces a marked Ca^{2+} influx through an effective activation of the P_{2x}-purinoceptors. This amplitude of the ATP-induced rise of $[Ca^{2+}]_i$ suggests thus that neither the breakdown of ATP nor the tachyphylaxis of the P_{2x}-purinoceptors account for the low contractive effect of ATP.

TES induced strong contractions in the mouse bladder. A force decline was already observed within 15 s while $[Ca^{2+}]_i$

in contrast remained elevated. Furthermore, in preparations preincubated with atropine, force induced by TES declined after 15 s, suggesting that a non-cholinergic mediator could be responsible for this Ca^{2+}-independent relaxation. Thus ATP, either exogenously or endogenously applied to the bladder smooth muscle, was associated with an early Ca^{2+}-force uncoupling. This observation suggested that ATP could bind to relaxing postsynaptic purinoceptors besides its activation of contracting P_{2x}-dependent purinoceptors. In order to reveal the possible relaxing effect of ATP, we analysed its effects in precontracted preparations.

In the carbachol-prestimulated bladder, ATP induced a biphasic force response, i.e. a marked contraction followed by a sustained relaxation (Figure 3). A biphasic effect of ATP has been reported in respiratory (Brown & Burnstock, 1981), vascular (Ralevic & Burnstock, 1991), digestive (Manzini *et al.*, 1985; Lefebvre & Burnstock, 1990) and genital (Boland *et al.*, 1992) smooth muscles, but to our knowledge not yet in the urinary bladder. The contractile effects of ATP were more pronounced in the carbachol-prestimulated bladder than under resting conditions. The mechanism underlying this increased potency of ATP for contraction is not known but could involve either a postsynaptic synergism between carbachol and ATP at the muscarinic receptor and the P_{2x}-purinoceptors as observed between noradrenaline and ATP for the vas deferens (Witt *et al.*, 1991) or an intracellular agonist-induced increase by carbachol of the sensitivy of the contractile filaments to Ca^{2+} (Himpens *et al.*, 1990).

In smooth muscle preparations containing both contracting and relaxing purinoceptors, the force response to ATP is the balance between two opposite effects. Here, during superfusion with 100 or 1000 μM ATP, the P_{2x}-dependent contractile effect predominated. In order to unmask the relaxation to ATP, we examined its effects in two conditions which are supposed to inhibit the P_{2x}-contraction. The first condition was to apply ATP twice successively in order to induce the tachyphylaxis which is specific for the P_{2x}-purinoceptors (Kennedy, 1990). In this condition, the ATP-induced rises of $[Ca^{2+}]_i$ and of force were decreased and the relaxation was more pronounced, i.e. the force level then declined close to the resting value. The decrease of the Ca^{2+}- and the contractile responses to the second ATP application can be explained by tachyphylaxis of the P_{2x} purinoceptors. The finding that relaxation induced by ATP in this condition did not decrease suggests that the two opposing effects are mediated through two different purinoceptors. The second experimental condition was to apply ATP in the K^+-depolarized bladder smooth muscle, in which the depolarizing effect of the P_{2x}-purinoceptors is expected to be markedly reduced, as well as their contractile effect. In fact, in the K^+-depolarized preparation, ATP induced a rise of $[Ca^{2+}]_i$ which was 56% of that observed in the carbachol-precontracted preparation. The contraction elicited by ATP was hardly observed, and was followed by a marked relaxation. In the two above mentioned experimental conditions, the relaxation induced by ATP occurred within about 30 s at an elevated $[Ca^{2+}]_i$, was sustained during the ATP application, and was reversible on washing out of ATP.

The relaxing purinoceptors in the smooth muscle are subclassified the P_{2y} and the A_1 adenosine receptor of the P_1-purinoceptor (Burnstock & Kennedy, 1985; Kennedy, 1990). Relaxing P_1-purinoceptors were reported in neonatal rat urinary bladder (Nicholls *et al.*, 1990) and are potently inhibited by 10 μM 8-phenyltheophylline (Griffith *et al.*, 1981). Our finding that the ATP-induced relaxation was not inhibited by 8-phenyltheophylline suggests that ATP acts on P_2-purinoceptors by itself, and not by its breakdown products on P_1-purinoceptors (Moody *et al.*, 1984). The P_2-type of the bladder relaxing purinoceptor activated by ATP is also supported by the observed rank order of potency for relaxation of the ATP analogues, which was characteristic for the P_{2y}-purinoceptors (Kennedy, 1990). Similar findings were recently reported in the mouse vas deferens smooth muscle (Boland *et al.*, 1992). To our knowledge, the relaxing effect of ATP has never been reported in the rodent bladder.

The physiological function of these relaxing purinoceptors is unknown. These inhibiting receptors could be activated *in vivo* during micturition, as suggested by the early relaxation observed during *in vitro* TES-induced stimulation. On the other hand, the continuous basal release of ATP (Burnstock *et al.*, 1978) could preferentially activate the relaxing purinoceptors during the bladder filling, thereby preventing the muscle contraction. The presence of two subtypes of purinoceptors in the bladder, mediating opposite mechanical effects, can explain both the striking Ca^{2+}-force dissociation induced by ATP and its low contractile potency.

In conclusion, besides its contractile effect through P_{2x}-purinoceptors, ATP also activates relaxing P_{2y}-purinoceptors in the mouse and other rodent bladder smooth muscles. It could be hypothesized that they counteract the bladder contraction during micturition or that they induce smooth muscle inhibition during the bladder filling. This latter hypothetical effect could explain in part the high compliance of the bladder smooth muscle.

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