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ATP activates P_{2x}-contracting and P_{2y}-relaxing purinoceptors in the smooth muscle of mouse vas deferens

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1 The mechanism for the low potency of exogenous ATP in producing contraction at the P_{2x}-purinoceptors in the smooth muscle of the mouse vas deferens (VD) was examined.

2 The measure of the breakdown of ATP in contact with the VD showed that its degradation was limited and did not account for its weak contractile effect.

3 Externally applied, ATP induced a small and transient contraction but a marked and prolonged increase of the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which suggests an efficient binding to the P_{2x}-purinoceptors. Such a calcium-force dissociation was not observed with β,γ-methylene ATP (β,γ-Me-ATP), a structural ATP analogue.

4 The force response of precontracted VD to ATP was biphasic, consisting of a small initial contraction followed by a sustained marked relaxation. In contrast, β,γ-Me-ATP elicited a pronounced contraction without ensuing relaxation.

5 ATP was more potent than adenosine in producing relaxation, and the relaxation was not antagonized by 8-phenyltheophylline, suggesting the activation of P_2-purinoceptors.

6 For this relaxation, the rank order of potency was 2-methyl-thio-ATP (2-MeSATP) > ATP > β,γ-Me-ATP, which is characteristic for the P_{2y}-purinoceptors.

7 Reactive Blue 2, a P_{2y}-purinoceptor antagonist, was found to reduce the relaxation mediated by ATP.

8 These results indicate that ATP acts in VD not only on contracting but also on relaxing P_2-purinoceptors, eliciting thereby overlapping opposite effects. In VD, the classical low potency of ATP for contraction is thus not explained by its low bioavailability or its low binding, but rather by its low specificity for the contracting P_{2x}-purinoceptors, leading to the activation of the relaxing P_{2y}-purinoceptors.

Keywords: ATP: P_2-purinoceptors; cytoplasmic Ca^{2+} concentration; smooth muscle, vas deferens

Introduction

In 1978, Burnstock introduced a classification of purinoceptors into P_1- and P_2-types. At the P_1-purinoceptors, adenosine acts with greater potency than adenosine 5'-triphosphate (ATP) and the response is inhibited by theophylline. At the P_2-purinoceptors, however, ATP is more potent than adenosine and not antagonized by theophylline. The P_2-purinoceptors have been further subdivided into major classes, the P_{2x}- and P_{2y}-subtypes (Burnstock & Kennedy, 1985), mediating in smooth muscles contracting and relaxing effects, respectively. These two subtypes are mainly distinguished by the rank order of potency for the contractile response of substituted analogues of ATP, i.e. β,γ-methylene-ATP (β,γ-Me-ATP) and 2-methyl-thio-ATP (2-MeSATP). At the contracting P_{2x}-purinoceptor, the characteristic rank order of potency is β,γ-Me-ATP > ATP ⩾ 2-MeSATP. An inverse order is observed at the relaxing P_{2y}-purinoceptor. Other clues supporting the existence of P_2-purinoceptor subtypes (Kennedy, 1990) are the desensitization occurring at the P_{2x}- but not at the P_{2y}-purinoceptor, and the specific antagonism of Reactive Blue 2 for the P_{2y}-purinoceptor. The classification of smooth muscle P_1- and P_2-purinoceptors is mainly based on mechanical studies, the interpretation of which remains complex because of both the possible extracellular breakdown of nucleotides and the heterogeneity of purinoceptors present in a given preparation. This may lead to simultaneous interactions between various agonists and different receptors. A study of the post-receptor signal of P_{2x}-purinoceptors would clarify the biochemical effects of purines in the smooth muscles.

The contracting P_{2x}-purinoceptor was initially described in the vas deferens (Fedan *et al.*, 1982; Burnstock *et al.*, 1985) and is still considered to be the only type of postsynaptic purinoceptor activated by ATP in this tissue. However, the vas deferens contractile response to exogenous ATP is very weak (Fedan *et al.*, 1982; Hourani *et al.*, 1986; Wilkund & Gustafsson, 1988; von Kügelen *et al.*, 1990). We used the mouse vas deferens (VD) to examine three hypotheses which could explain the low potency of ATP at the contracting P_{2x}-purinoceptors; low bioavailability, low binding or low specificity. To clarify the question of the agonist bioavailability, we measured the breakdown of ATP in contact with VD. To test the binding of ATP to the P_{2x}-purinoceptors, we determined the amplitudes of its post-receptor cytosolic messenger, i.e. of the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). To investigate whether the low potency of ATP in producing contraction could result from its simultaneous binding to an unknown relaxing purinoceptor, we applied ATP to precontracted VD. The results reported here lead us to the conclusion that both P_{2x}- and P_{2y}-purinoceptor subtypes are present in the VD smooth muscle cells, and that the weak and transient contractile effect of ATP probably results from the opposite effects of its binding to contracting P_{2x}- and relaxing P_{2y}-purinoceptors.

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Methods

Muscle tissues

Adult male albino mice (NMRI, 3–4 months old, 30–40 g) were killed by cervical dislocation after anaesthesia with ether. The vas deferens (VD) was removed and transferred to the oxygenated HEPES-buffered Krebs solution. The 10 mm end segment of its prostatic part was isolated, dissected free from surrounding tissues and opened longitudinally in order to remove its epithelial layer by gentle rubbing. Histological analysis of these VD strips showed that they consisted of a thick smooth muscular wall of about 20 cell layers, surrounded by poorly developed interstitial tissue. We also confirmed that the epithelial layer had been completely scraped away. Histological sections showed that smooth muscle represented more than 95% of the cells of the preparation.

Analysis of purine concentrations

The concentrations of adenosine, AMP, ADP and ATP generated at room temperature after application of purines on the VD muscle (4.82 ± 0.38 mg, $n = 12$) in the 1 ml chamber were measured by high performance liquid chromatography (h.p.l.c.). The stock solution of ATP contained 1.6% ADP, 0.2% AMP and no detectable adenosine. The stock solution of adenosine contained 0.05% AMP and no detectable ADP or ATP. VD strips were incubated for 60 min in 1 ml Krebs solution, as a control for possible spontaneous endogenous release of purines by the muscle. As control for non-enzymatic breakdown, ATP was incubated for 30 min in 1 ml HEPES-Krebs solution without any contact with VD. Adenosine was incubated for 15 min with VD to measure its possible uptake by the muscle. The breakdown of purines was determined by applying ATP and β,γ-Me-ATP to VD into the chamber, and collecting samples of the medium at the surface of the VD after 3 s, 30 s, 2 min, 5 min and 15 min incubation. Aliquots (100 μl) of the medium were transferred into 25 μl of ice-cold 10% (w/v) $HClO_4$. After 15 min at 4°C, the extracts were neutralized with 3 M-KOH/3 M-$KHCO_3$ and stored at −20°C until the assays were performed. Adenylic nucleotides (ATP, ADP and AMP) were measured by anion exchange h.p.l.c. on a 100 × 4.7 mm PartiSphere 5 SAX anion exhange column (Whatman, Maidstone, Kent) using a gradient from 0.01 M $NH_4H_2PO_4$ at pH 3.7 to 0.48 M $NH_4H_2PO_4$ at pH 3.7 over 30 min at a flow rate of 2 ml min^{-1}. The adenylic nucleoside (adenosine) was measured by reversed phase h.p.l.c. on a 110 × 4.7 mm PartiSphere 5 C_{18} column (Whatman, Maidstone, Kent) eluted over 15 min at a flow rate of 1.4 ml min^{-1} with 0.01 M NaH_2PO_4 at pH 5.5, and a 0 to 20% gradient of methanol/H_2O (1/1).

Simultaneous measurements of $[Ca^{2+}]_i$ and force

VD was loaded for 3 h with 2 μM fura-2AM, a fluorescent Ca^{2+}-indicator, in the normal HEPES-buffered Krebs solution at room temperature. This procedure did not affect either the amplitude or the kinetics of the force response to 140 mM K^+ or to purine agonists ($n = 6$). After loading, VD was rinsed for 30 min and mounted in a 1 ml chamber under isometric conditions. VD was stretched to a steady passive tension of 2 mN and allowed to equilibrate for 30 min before addition of drugs. VD was superfused at a constant flow rate of 4 ml min^{-1}. The mounting of VD in the chamber (Boland *et al.*, 1992) and the apparatus used for the simultaneous measurements of the fura-2 fluorescence and the force response in intact smooth muscle strips (Himpens & Somlyo, 1988) have been described. An internal calibration of the fluorescent signals was performed at the end of each experiment to determine the value of the minimal (R_{min}) and the maximal (R_{max}) ratio, using the procedure designed by Himpens *et al.* (1988). $[Ca^{2+}]_i$ values are routinely expressed in nM in the text and on the original traces. The force response is expressed either in mN or as a percentage of the maximal force level (100%) obtained in VD during initial stimulation with high K^+ solution, as usually used for the vas deferens (Fedan *et al.*, 1982; Burnstock *et al.*, 1985; Hourani *et al.*, 1986). The passive tension of 2 mN is used as the basal force reference (0%). For the study of the effect of the purine antagonists (see Figure 5), the relaxation by ATP was normalized to the raised-tone level elicited by prolonged stimulation with the 140 mM K^+ solution. Owing to the limited supply of the ATP analogues, perfusions with high concentrations could not be used to obtain maximal responses. Because of the marked desensitization occurring at the P_2-purinoceptors, only one concentration of one purine analogue was applied on each VD strip, except for the study of the antagonism of the purinoceptors where a second application of the agonist was performed in the presence of 8-phenyltheophylline (8PT) or Reactive blue 2 (RB2), 20 min after washing out the control agonist application. Neither ATP nor its analogues used here were autofluorescent. However, both 8PT and RB2 increased the fluorescent signals at 510 nm, especially after excitation with 340 nm, and thereby affected the 340/380 ratio values and the estimated $[Ca^{2+}]_i$. This interference made it impossible to determine $[Ca^{2+}]_i$ in their presence.

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. In the Ca^{2+}-free Krebs solution, $CaCl_2$ was omitted and 2 mM EGTA was added. Isotonic high K^+ solutions (70 and 140 mM $[K^+]_o$) were obtained by replacing external Na^+ by an equivalent amount of K^+. Sodium salt of adenosine triphosphate (ATP), sodium salt by β,γ-methylene ATP (β,γ-Me-ATP), hemisulphate salt of adenosine, phenylephrine and Reactive blue 2 (Cibacron blue 3GA, 55%) were obtained from Sigma. Tetrasodium salt of 2-methylthio-adenosine triphosphate (2-MeSATP) was obtained from ICN Biochemicals (Cleaveland, Ohio, USA). Isoprenaline hydrochloride was from Winthrop Lab. (Brussels, Belgium). 8-phenyltheophylline and Ionomycin were from Calbiochem. Fura-2AM was from Molecular Probes (Eugene, OR, U.S.A.). All other reagents were of analytical grade. 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).

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 using Student's t test (paired two-tailed t test). A probability of less than 0.05 was considered significant.

Results

Extracellular metabolism of purines

Incubation of the VD strips in 1 ml HEPES-Krebs solution for 30 min did not lead to detectable levels of purines in the bathing medium ($n = 4$), indicating that the release of purines by VD was not significant. After 15 min of application of 1000 μM adenosine on the VD in the chamber, its concentration did not decrease ($n = 2$). This result excludes a significant lowering of the adenosine concentration due to nucleoside uptake by VD. Application of 100 μM β,γ-Me-ATP, a slowly degradable structural ATP analogue (Fedan *et al.*, 1982), did not result in the occurrence of any detectable

breakdown products ($n = 2$). Incubation of 100 μM ATP alone for 60 min in 1 ml HEPES-Krebs solution, without contact with VD, was not associated with purine degradation ($n = 3$). Exposure of VD to ATP was followed by a time-dependent accumulation of breakdown products, i.e. ADP, AMP and adenosine. Aliquots in contact with the VD were collected after 3 s, 30 s, 2 min, 5 min and 15 min of ATP application. After application of 100 μM ATP for 5 min ($n = 2$), the concentrations of ATP and ADP (P_2-agonists) were 75 and 12 μM, respectively. Under these conditions, AMP and adenosine (P_1-agonists) were 5 and 2 μM. As can be seen in Figure 1, after 5 min contact between VD and 1000 μM ATP, the concentrations of ATP and ADP were 953 and 56 μM, respectively, while the concentrations of AMP and adenosine were 15 and 13 μM, respectively. Preincubation of VD with 100 μM AOPCP, a specific inhibitor of the ecto 5′-nucleotidase (Burger & Lowenstein, 1975), which is the enzyme involved in the extracellular degradation of AMP to adenosine, modified the concentration changes in AMP and adenosine, which were respectively 19 and 6 μM after 5 min application of 1000 μM ATP ($n = 2$). Thus, whatever the ATP concentration, the concentration of P_1-agonists remained low, while the ATP concentration did not decrease to less than 75%. The bioavailability of ATP for the purinoceptors was therefore only slightly impaired.

Effects of ATP on $[Ca^{2+}]_i$ and force

VD does not present basal tonus or spontaneous contractile activity (Boland *et al.*, 1992). In the resting condition, $[Ca^{2+}]_i$ was 103 ± 5 nM ($n = 21$). Superfusing VD with a depolarizing solution containing 140 mM K^+ and 1.5 mM Ca^{2+} (Figure 2a) induced a maximal increase of $[Ca^{2+}]_i$ to 433 ± 44 nM ($n = 21$) and of force to 15 ± 1 mN. These values will be used further on as the 100% reference, for $[Ca^{2+}]_i$ and force, respectively. We studied the effects of superfusion for 5 min at 4 ml min^{-1} with purinoceptor agonists (100 μM) on $[Ca^{2+}]_i$ and force level in VD. ATP elicited an increase of $[Ca^{2+}]_i$ up to 361 ± 48 nM ($n = 9$) but force hardly rose (by 3 ± 1%, Figure 2a) and returned to its basal level within 1 min while $[Ca^{2+}]_i$ was still at 235 nM ($n = 9$). The amplitude of this ATP-induced $[Ca^{2+}]_i$ transient suggests that the transduction process worked efficiently in VD. When the superfusion with 100 μM ATP was repeated at 5 min intervals, the peak of $[Ca^{2+}]_i$ declined by 65 ± 3% at the second and the third stimulation (Figure 2b), while the force level remained at its basal level ($n = 4$). This desensitization was specific for the purinoceptors because 100 μM phenylephrine elicited, after 3 such successive ATP applications, an increase of $[Ca^{2+}]_i$ which was 92 ± 11% ($n = 4$) of the one it induced before the ATP applications ($n = 4$). These findings suggest that the involved purinoceptors displayed tachyphylaxis. However, the kinetics of this coupling could not be studied at the rather slow superfusion procedure. Therefore, we injected ATP (final concentration 100 μM) over a period of about 100 ms, close to the muscle (Figure 3). The latency period between the ATP injection and the $[Ca^{2+}]_i$ rise was 0.32 ± 0.8 s and $[Ca^{2+}]_i$ peaked after 4.5 ± 0.4 s, while contraction reached 13 ± 1% of the 140 K^+ reference. Finally, to study the dependence of the ATP-induced Ca^{2+} transient on the external Ca^{2+}, we applied ATP after 15 min perfusion with a Ca^{2+}-free Krebs solution containing 2 mM EGTA. Under these conditions, $[Ca^{2+}]_i$ decreased to 65 ± 8 nM, as previously reported (Boland *et al.*, 1992) and 100 μM ATP only increased $[Ca^{2+}]_i$ to 86 ± 18 nM ($n = 4$), without inducing any force (not shown).

The effects of ATP were compared to those induced by superfusion with its substituted analogue. Superfusion with 100 μM 2-MeSATP ($n = 4$) induced an increase of $[Ca^{2+}]_i$ and of force comparable to those elicited by ATP (not shown). However, β,γ-Me-ATP increased $[Ca^{2+}]_i$ to 187 ± 13 nM ($n = 7$) and force up to 27 ± 8% (Figure 2a). The two parameters progressively declined after 5 min stimulation

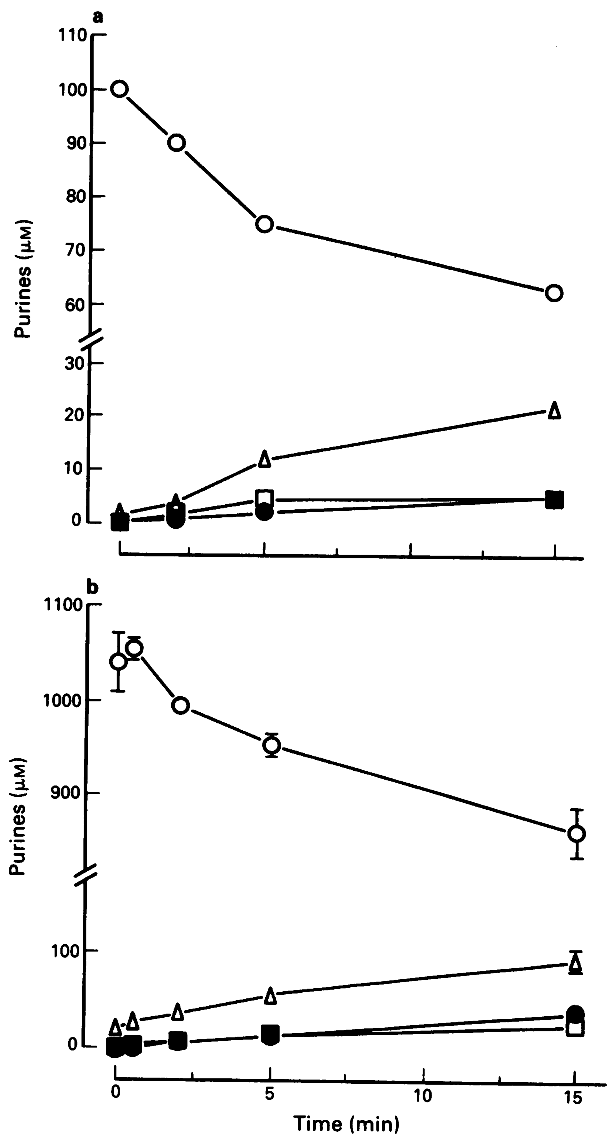

Figure 1 Time-course of the breakdown of ATP (○) into ADP (△), AMP (□) and adenosine (●) by the vas deferens (VD). ATP was applied to the mouse VD at a final concentration of (a) 100 μM ($n = 2$) and (b) 1000 μM ($n = 3$) in the chamber containing 1 ml HEPES-Krebs solution. Samples of the medium were taken at the VD surface at the times shown and further analysed by h.p.l.c.

with β,γ-Me-ATP to 130 ± 10 nM and 15 ± 5%, respectively. Adenosine 100 μM ($n = 4$) did not alter either $[Ca^{2+}]_i$ or force. This indicates that P_1-purinoceptors are not involved in the contractile response or in the Ca^{2+} changes in VD. So far, our results on the potency for contraction and on the tachyphylaxis indicate that P_{2x}-purinoceptors mediate the ATP-induced contraction in VD. The paradoxical dissociation, both in kinetics and amplitude, between the increases of $[Ca^{2+}]_i$ and of force is a puzzling finding. The respective evolutions of the prolonged and high $[Ca^{2+}]_i$ rise and the transient and small force contraction evoked by ATP suggest the presence of an unknown relaxing process. Because of the absence of basal tonus in VD, this relaxation could have been masked. We therefore applied purines on the precontracted VD to investigate this hypothesis.

Effects of ATP in the precontracted preparations

Non-purine agonists (noradrenaline, carbachol, histamine) in concentrations up to 1 mM failed to induce in VD a sustained

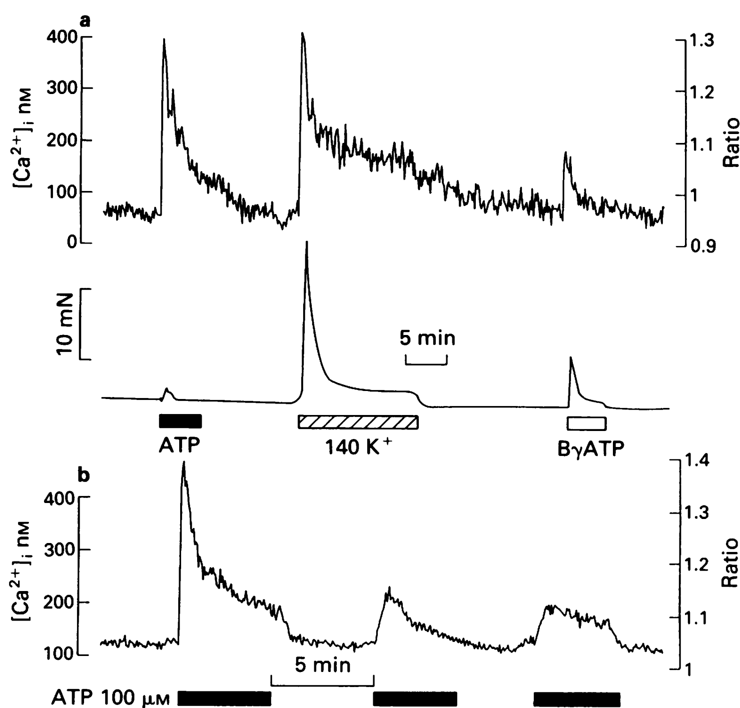

Figure 2 Panel (a) shows the changes in $[Ca^{2+}]_i$ (upper trace) and force (lower trace) in the vas deferens in response to superfusion with 100 μM ATP (solid bar) or 100 μM β,γ-methylene-ATP (β,γ-Me-ATP, open bar). Maximal increases (100%) of $[Ca^{2+}]_i$ and of force were obtained after 1 min depolarization with 140 mM K^+ (hatched bar). Results were independent of the sequence of perfusion with ATP, β,γ-Me-ATP and 140 K^+. In panel (b), for study of receptor tachyphylaxis, 100 μM ATP was superfused three times for 5 min at 5 min intervals.

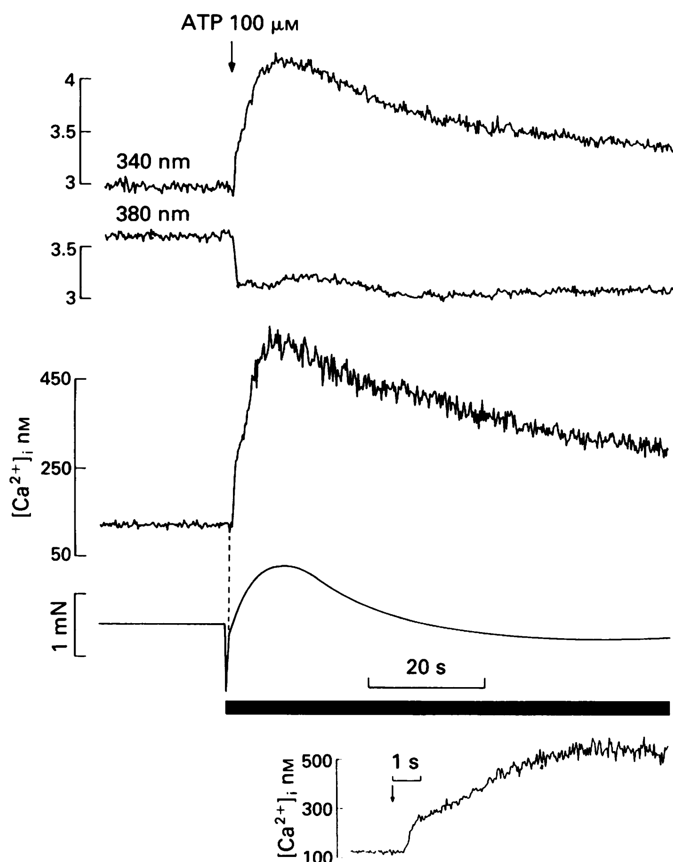

Figure 3 Effect of direct injection in the medium of 100 μM ATP on the fluorescent signals (upper traces), the $[Ca^{2+}]_i$ (middle trace) and the force (lower trace) of the vas deferens. The sampling frequency was 100 Hz. To obtain a precise time reference, a negative signal applied to the transducer was suddenly removed at the time of the ATP injection. The methodological imprecision was lower than 100 ms. In the inset, the same ATP stimulation is displayed at a higher time resolution, showing that the increase of $[Ca^{2+}]_i$ began within less than 500 ms after the ATP injection (arrow).

contraction. However, prolonged depolarization with 140 mM K^+ induced a sustained contraction (Figure 2a). After 15 min stimulation, $[Ca^{2+}]_i$ was still at 247 ± 15 nM, and force at $18 \pm 2\%$ of the initial peak reference value ($n = 21$). Under these conditions, no force change was induced by superfusion with 10 μM ATP or 10 μM adenosine, while 10 μM 2-MeSATP produced a slight but significant relaxation to $16 \pm 1\%$. Next, we superfused purines for 5 min at 100 μM concentration. ATP elicited a rise in $[Ca^{2+}]_i$ up to 417 ± 71 nM ($n = 6$) (Figure 4a). This parameter returned within 2 min to the previous level (257 ± 20 nM), while the force response presented after 30 s a relaxation to $14 \pm 0.6\%$, following an initial variable and small contraction to $20 \pm 0.7\%$. After washing out ATP, force recovered its previous level (18%). Changes in $[Ca^{2+}]_i$ and force induced by 2-MeSATP (Figure 4a) were comparable to those obtained by ATP ($n = 3$). β,γ-Me-ATP (Figure 4a) increased $[Ca^{2+}]_i$ to 320 ± 30 nM ($n = 6$) and of force to $31 \pm 2\%$, and within 2 min, both $[Ca^{2+}]_i$ and force recovered their previous levels. No relaxation below the precontracted force level was thus observed. Adenosine 100 μM did not significantly alter either $[Ca^{2+}]_i$ or force ($n = 5$), except in 2 preparations in which force decreased slightly to 17%.

The biphasic pattern of the ATP-induced force response became more pronounced at 1000 μM ATP. Force initially rose to $23.2 \pm 3\%$ and thereafter relaxed to $6.3 \pm 1\%$ ($n = 14$) (Figure 4b). In order to study the purinoceptor tachyphylaxis, we also superfused the tissue with 1000 μM ATP twice at 5 min intervals ($n = 4$). In this condition, we could not observe the initial contraction during the second ATP application (Figure 4b), while the relaxation was similar to that obtained during the first ATP stimulation. Superfusion of VD with a mixture of the concentrations of the P_1-agonists as produced by the breakdown of 1000 μM ATP, i.e. with 15 μM AMP and adenosine (see Figure 1), induced only a relaxation to $17 \pm 0.5\%$ ($n = 4$). Adenosine, 1000 μM relaxed VD to $10 \pm 1\%$; isoprenaline, 100 μM, ($n = 5$) and papaverine, 1000 μM, ($n = 4$) produced relaxation to 9 ± 1 and to $6 \pm 2\%$, respectively. The relaxing effect of ATP is not species specific: in the rat ($n = 6$) and the guinea-pig ($n = 6$) vas deferens, 1000 μM ATP also induced a biphasic force response consisting of a small initial contraction followed by a steady relaxation (data not shown).

To characterize further the receptor involved in the relaxation induced by ATP, we studied the effect of 8PT, a potent P_1-antagonist (Griffith et al., 1981) and of RB2, a selective (Burnstock & Warland, 1987) but weak P_{2y}-antagonist, during non-cumulative applications for 5 min of 100, 250, 500 or 1000 μM ATP. The results are presented in Figure 5 and the relaxation is expressed in these experiments as a percentage of the steady raised-tone induced by 140 mM K^+. The concentration-response curve in the absence of the antagonist is represented by circles. A preceding incubation of VD for 15 min with 10 μM 8PT (triangles) did not significantly modify the relaxation by ATP up to 1000 μM. This 8PT concentration is reported to inhibit by about 50% the relaxation induced by 1000 μM ATP in the histamine-contracted rabbit central ear artery (Kennedy & Burnstock, 1985), in which the relaxation by ATP is P_1-dependent. We observed an inhibition of 60% of the ATP-induced relaxation in this rabbit preparation ($n = 5$), indicating that our 8PT solution was effective. Also 100 μM 8PT did not inhibit the ATP-induced relaxation in VD, but it should be mentioned that at this concentration, not all 8PT was dissolved. Incubation of VD for 15 min with 200 μM RB2 did not produce by itself any force decline. RB2 slightly but significantly inhibited the relaxation by ATP (squares), but not that caused by 100 μM isoprenaline ($n = 4$) or by 1000 μM papaverine ($n = 3$). RB2 200 μM did not affect the force response to ATP in 25% of preparations. In the precontracted VD incubated with 200 μM RB2, 1000 μM ATP elicited an initial contraction to $29 \pm 2\%$, which is higher than the value induced by the same ATP solution in the absence of RB2 (see above).

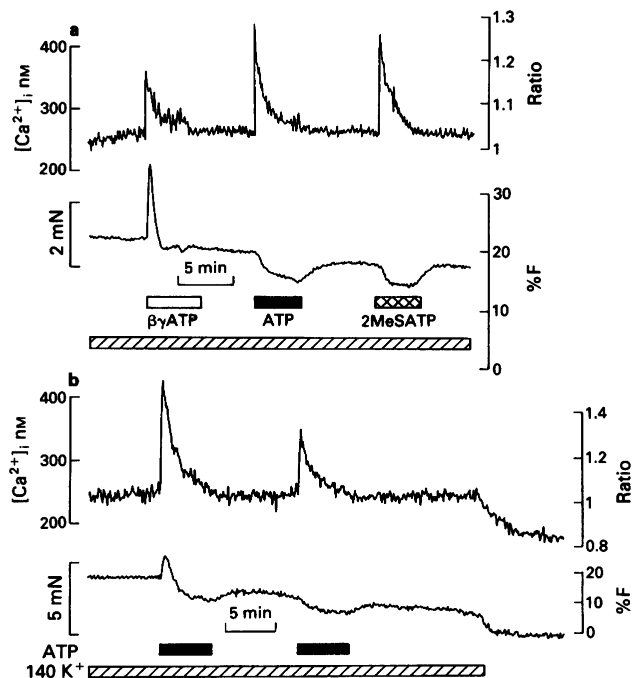

Figure 4 (a) Comparison of the effect of β,γ-methylene-ATP (β,γ-Me-ATP) (open bar), ATP (solid bar) and 2-methyl-thio-ATP (2-MeSATP) (cross hatched bar) on $[Ca^{2+}]_i$ (upper trace) and on force (lower trace) in the K^+-precontracted vas deferens (VD). β,γ-Me-ATP induced a contraction, while ATP and 2-MeSATP elicited marked relaxation after a small and initial contraction. Here, the stimulation with β,γ-Me-ATP produced a P_{2x}-purinoceptor tachyphylaxis so that the initial contraction during the following ATP application was absent. (b) Shows the effect of 1000 μM ATP on $[Ca^{2+}]_i$ (upper trace) and the force (lower trace) in K^+-precontracted VD. The first ATP application always triggered a pronounced and transient rise in $[Ca^{2+}]_i$ and a biphasic force pattern consisting of an initial contraction followed by a steady relaxation. A second ATP application induced a lower rise of $[Ca^{2+}]_i$ and a loss of the early contraction, suggesting tachyphylaxis of the P_{2x}-purinoceptors. The relaxation was reproducible and was not associated with a detectable decline in $[Ca^{2+}]_i$.

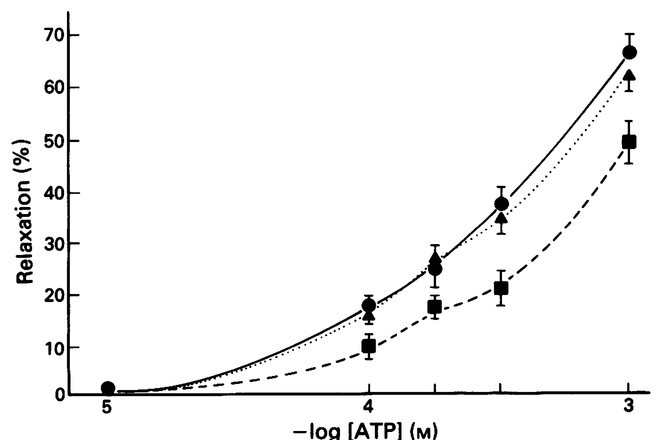

Figure 5 Effects of the antagonist of P_1-purinoceptor, 8-phenyltheophylline (10 μM) (▲), and of P_{2y}-purinoceptor, Reactive blue 2 (200 μM) (■), on the relaxation by ATP. Relaxation is expressed as a percentage of the maintained tone obtained in the vas deferens by a prolonged stimulation with 140 mM K^+; (●) represent the controls.

Discussion

We describe here the first study of the simultaneous measurements of the Ca^{2+}-transients and the force responses induced by ATP and related nucleotides in the intact vas deferens. It is generally accepted that ATP is less potent than β,γ-Me-ATP in activating the contracting P_{2x}-purinoceptors of the vas deferens, because ATP is enzymatically broken down or because it presents a low binding to its receptor. Although the breakdown observed at the tissue surface might be an underestimate of the actual breakdown occurring at the receptor level, our measurements rule out a significant enzymic degradation of ATP in the VD. Similar findings were reported in the taenia coli in which no correlation was found either between the ATP analogue potency for contraction and its rate of degradation (Welford *et al.*, 1986). However, we observed a very large ATP-induced rise in $[Ca^{2+}]_i$.

In the vas deferens smooth muscle, the P_{2x}-purinoceptors trigger the opening of non-selective ion channels associated to membrane depolarization and early Ca^{2+} influx (Friel, 1988). Thus an ATP-induced Ca^{2+} signal mediated through the P_{2x}-purinoceptors is expected to be strongly dependent on the external Ca^{2+} and to occur relatively fast. The activation of the P_{2y}-purinoceptors in endothelial cells (Carter *et al.*, 1988) and in erythrocytes (Boyer *et al.*, 1989; Cooper *et al.*, 1989), can also lead to an elevation of $[Ca^{2+}]_i$, but through an IP_3-induced Ca^{2+}-release independent of the extracellular Ca^{2+} and delayed by several seconds (Boeynaems & Pearson, 1990). Here, we describe the marked inhibition of the ATP-induced Ca^{2+} signal in the Ca^{2+}-free solution and its short delay (320 ms). These characteristics make the participation of P_{2y}-purinoceptors in the Ca^{2+} signal triggered by ATP less likely, and suggest that the ATP-induced Ca^{2+} signal was mainly due to the activation of P_{2x}-purinoceptors. Moreover, the P_{2y}-induced Ca^{2+}-dependent relaxation observed in digestive smooth muscle is caused by membraneous hyperpolarization (Crist *et al.*, 1992). The finding that ATP relaxed K^+-depolarized preparations in our experiments suggests that another pathway is involved in the VD and provides an additional argument against the P_{2y} participation in the ATP-induced rise of Ca^{2+}. As indicated by electrophysiological measurements of the induced inward current (Friel, 1988), the potency of ATP is much higher than that of β,γ-Me-ATP at the P_{2x}-purinoceptor. The resulting membranous depolarization activates Ca^{2+}-influx (Kennedy, 1990). Our measurement of the rise of $[Ca^{2+}]_i$ confirms that ATP is more potent than β,γ-Me-ATP at the P_{2x}-purinoceptors. Taken together, these findings suggest that the difference in Ca^{2+} rise observed between ATP and β,γ-Me-ATP is mainly due to different potencies at the P_{2x}-purinoceptors. It is therefore not necessary to invoke another mechanism such as a P_{2y}-associated rise of Ca^{2+} to explain the observed rank order of potency for the Ca^{2+}-increase (ATP = 2-MeSATP > β,γ-Me-ATP).

We applied ATP to precontracted VD to unmask a possible relaxant effect and we observed a superimposed biphasic force response consisting of a small and variable initial contraction followed by a maintained relaxation. The small initial contraction showed marked tachyphylaxis and a rank order of potency (β,γ-Me-ATP > ATP $\geq$ 2-MeSATP), which is typical for the P_{2x}-purinoceptors (Kennedy, 1990). The relaxation by ATP was not inhibited by 8PT and was more pronounced than that due to adenosine, indicating that the relaxation was P_2-dependent. The breakdown products of ATP which are P_1-agonists induced less than 10% of the relaxation produced by ATP. Therefore, ATP must act mainly by itself on VD P_2-purinoceptors. The P_{2y}-mediation of the relaxation by ATP was supported by the observed rank order of potency for relaxation (2-MeSATP $\geq$ ATP > β,γ-Me-ATP) and by the specific antagonism by RB2 (Manzini *et al.*, 1985; Burnstock & Warland, 1987; Lefebvre & Burnstock, 1990). The findings that successive ATP applications led to tachyphylaxis of the contractile response but not of the relaxation (Figure 4b) and that RB2 induced in the precontracted VD both an increase of the contraction and an inhibition of the relaxation in response to ATP, suggest that the contracting and the relaxing effects are mediated through different receptors. Such a biphasic force response to ATP has previously been reported in the guinea-pig trachea (Brown & Burnstock, 1981) and in contracted vascular preparations (Ralevic & Burnstock, 1991). The force response to ATP thus seems to be the result of two opposite effects in preparations expressing both P_{2x}- and P_{2y}-purinoceptors, while that caused by β,γ-Me-ATP depends mainly on P_{2x}-activation. Substitution of a methylene bridge in the polyphosphate chain of ATP indeed increases the specificity for P_{2x}-purinoceptors and reduces its potency in activating the relaxing P_{2y}-purinoceptors (Gordon, 1986): in precontracted VD, β,γ-Me-ATP produced a large transient contraction but did not evoke a subsequent relaxation, as previously observed in guinea-pig smooth muscle preparations containing P_{2y}-purinoceptors, i.e. trachea (Brown & Burnstock, 1981), taenia coli and aorta (Hourani *et al.*, 1985).

It can therefore be proposed that in the VD, ATP acts also on P_{2y}-purinoceptors, inducing a relaxation that can overcome the contractile effect mediated by the P_{2x}-purinoceptors. Binding of ATP at the activating P_{2x}-purinoceptors indeed directly activates ion channels (Friel, 1988), while binding at the inhibiting P_{2y}-purinoceptors elicits a slower G protein-dependent enzymic activation (Kennedy, 1990). This might lead to a difference in time-course between the opposite influences of activating and inhibiting processes. The fast transduction through P_{2x}-purinoceptors would first trigger muscle activation, but the combined effect of the P_{2x}-desensitization and the activation of the inhibiting P_{2y}-purinoceptors (which do not desensitize) eventually turns the balance in favour of relaxation. In contrast, ATP induces in the digestive and the respiratory smooth muscles an initial transient relaxation followed by a sustained contraction. The delay of this latter P_{2x}-dependent contraction might be explained by its mediation through the rather slow prostaglandin pathway, as indicated by its abolition by indomethacin (Burnstock *et al.*, 1978; Brown & Burnstock, 1981; Manzini *et al.*, 1985; Lefebvre & Burnstock, 1990). In VD, indomethacin did not modify the force response to ATP, as previously reported (Fedan *et al.*, 1982; Wilkund & Gustafsson, 1988). The release of ATP from purinergic nerve terminals *in situ* will minimize the diffusion delay and favour the initial contraction. This might explain why transmural electrical stimulation produces contractions up to 60% of the reference peak K^+ stimulation in VD incubated in the presence of phentolamine (Boland *et al.*, 1992). Finally, our hypothesis can also readily explain the difference in potency of ATP analogues on the $[Ca^{2+}]_i$ rise and on force development. Even if β,γ-Me-ATP binds less tightly than ATP to the P_{2x}-purinoceptors, producing thereby a smaller rise of $[Ca^{2+}]_i$ (Figure 2a), its preferential binding to P_{2x}-purinoceptors has to result in a higher force response because the inhibitory P_{2y}-effect comes into play very little.

Our simultaneous study of $[Ca^{2+}]_i$ and force clearly shows that the binding of ATP to P_{2x}- and to P_{2y}-purinoceptors does not result in a competition at the level of the same messenger because ATP produced a large increase of $[Ca^{2+}]_i$ but a limited force development. The effect of the elevated $[Ca^{2+}]_i$ on the force is thus somehow antagonized by the activation of the P_{2y}-purinoceptors through a still unknown mechanism which might affect either the level of the myosin phosphorylation or the activity of proteins involved in the actin-myosin interaction, like caldesmon or calponin.

These results also indicate that the order of potency for VD contraction by the ATP analogues, i.e. the essential criteria for the P_2-purinoceptor subclassification, depends mainly on their specificity for the P_{2x}-purinoceptors. The unexpected purinoceptor heterogeneity observed in the vas deferens suggests that the order of potency could appreciably vary among smooth muscles according to differences in the

expression of the P_2-purinoceptor-subtype(s), and it could explain, at least partly, the increasing discrepancy reported in the classification of P_2-purinoceptors.

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