Post-receptor pathway of the ATP-induced relaxation in smooth muscle of the mouse vas deferens

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- 1 The post-receptor pathway of the ATP relaxant effect in K⁺-precontracted vas deferens smooth muscle (VD) was examined.
- 2 The relaxation to ATP was not antagonized either by 10 µM methylene blue, a cyclic GMP inhibitor, by 10 µM indomethacin, an inhibitor of prostaglandin synthesis or by 100 µM N^G-nitro-L-arginine, an inhibitor of NO production.
- 3 The Rp-diastereomer of adenosine 3':5'-cyclic monophosphorothioate (Rp-cAMPS) 200 µM, a competitive inhibitor of cyclic AMP significantly diminished the relaxant response to ATP.
- 4 Isoprenaline 10 µM, a β-adrenoceptor agonist, produced a sustained relaxation, inhibited by Rp-cAMPS, without a significant change in [Ca²⁺]_i, thereby mimicking the ATP-induced relaxant effect.
- 5 The level of the phosphorylated myosin light chain in the precontracted VD was significantly lowered by 1000 µM ATP.
- 6 ATP (1000 µM) and isoprenaline (10 µM) produced the same increase (+ 50%) of [cyclic AMP] when applied to a resting VD.
- 7 The effect of simultaneous increases of [Ca²⁺]_i and of [cyclic AMP] produced by externally applied ATP are discussed.
- 8 These results suggest that ATP-induced relaxation in K⁺-precontracted VD is mediated by the activation of adenylyl cyclase.

Keywords: ATP; P₂-purinoceptors; cyclic AMP; myosin phosphorylation; relaxation; smooth muscle; vas deferens

Introduction

Co-transmission of adenosine 5'-triphosphate (ATP) and noradrenaline was initially observed in the sympathetic nerves of the rodent vas deferens (Sneddon & Westfall, 1984). In this tissue, ATP induces contraction through the so called P_{2X}-purinoceptors (Fedan *et al.*, 1982; Burnstock *et al.*, 1985), the activation of which triggers opening of non-selective ion channels, membrane depolarization and calcium influx (Friel, 1988). The contractile effect of externally applied ATP is however very weak and transient (Fedan *et al.*, 1982; Hourani *et al.*, 1986; Wilkund & Gustafsson, 1988; von Kügelen *et al.*, 1990). We recently reported that, in the mouse vas deferens (VD), ATP induced a large rise of the cytoplasmic free calcium concentration ([Ca²⁺]_i), as large as that produced by massive depolarization with isotonic K⁺ solution. We also observed that ATP could induce relaxation of a K⁺-depolarized preparation. Therefore, it was suggested that the calcium-force dissociation could be due to the simultaneous binding of ATP to both P_{2X}-purinoceptors, mediating contraction and P_{2Y}-purinoceptors, mediating relaxation (Boland *et al.*, 1992). We describe here our attempts to identify the post-receptor signal for the ATP-induced relaxant response. Several possible messengers were excluded on the basis of pharmacological experiments. Results show that externally applied ATP not only produces an intracellular rise of [Ca²⁺]_i, but also of adenosine 3':5'-cyclic monophosphate (cyclic AMP). The latter seems to be the post-receptor signal for the relaxant response. However, as discussed, this may not be sufficient to explain fully the observed calcium-force dissociation induced by ATP when applied to the resting VD.

Methods

Muscle preparation and measurements of cytosolic Ca²⁺ concentration and of force

We essentially followed the procedures previously described (Boland *et al.*, 1992) except for fura-2 fluorescence measurements. Adult male albino mice (NMRI) of 3–4 months were killed by cervical dislocation after anaesthesia with ether. The end segment of the prostatic portion of the VD was isolated and opened longitudinally; its thick mucosal layer was then scraped away by gentle rubbing. VD preparations were stretched to 2 mN passive tension and isometric contraction force was measured by use of a strain gauge (type FLA-3-11, Tokyo Sokki Kenkyujo Co. Ltd). Isometric force was expressed in mN on the original traces. In the text, the force relaxation produced in raised-tone preparations by ATP or isoprenaline is normalized to the steady raised tone level maintained by prolonged stimulation with the high K⁺ solution. All experiments were performed at room temperature.

Fura-2 fluorescence was measured with inverted fluorescence microscopic equipment (from Nikon and Deltascan, PTI) where the emission intensity was collected at a 510 nm by a photon counting tube (PTI). Calculation of [Ca²⁺]_i was performed by internal calibration of the fura-2 fluorescence signal, according to a procedure designed by Himpens and co-workers (1988). In order to verify that the [Ca²⁺]_i rise upon application of ATP, essentially came from muscle cells and not from nerve endings (known to be numerous in VD; Swedin, 1971), we checked that this [Ca²⁺]_i rise was unaffected either after incubation with ω-conotoxin (1 µM, 30 min), a blocking agent specific for the neuronal calcium channels (McCleskey *et al.*, 1987) or after pharmacological destruction of nerve endings (Wakade, 1979). In the latter case, mice were injected intraperitoneally with 6-hydroxydopamine (6-OHDA), three times at two days interval. This treatment totally abolished the response to electrical stimulation.

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Measurement of cyclic AMP

The concentration of the cytoplasmic cyclic AMP was determined by radioimmunoassay, as described (Barnette *et al.*, 1989; McHenry *et al.*, 1991). After 5 min incubation in the experimental solution, VD strips were rapidly frozen in liquid nitrogen and homogenized in 1 ml of 6% trichloroacetic acid (TCA) on ice. Precipitated proteins were removed by 2000 g centrifugation for 15 min. TCA was removed from the supernatant by four extractions with water-saturated ether (3 ml), and the samples were evaporated. The measurement of cyclic AMP was by radioimmunoassay (Amersham kit, U.K.). The results are expressed in femtomoles per milligram (fmol mg⁻¹) of frozen muscle.

Determination of the myosin light chain phosphorylation

Phosphorylation of the 20 kDa myosin light chain (LC) was determined by two dimensional electrophoresis. The frozen VD preparations were ground in a mortar precooled with liquid nitrogen, in 300 µl 0.5 M perchloric acid. After thawing at room temperature, 400 µl perchloric acid was added and the whole extract was centrifugated for 5 min at 5000 r.p.m. The pellet was dissolved in 10 µl of the lysis buffer (9.5 M urea, 2% (w/v) nonidet P40 (NP 40), 1.6% ampholine pH 5–7, 0.4% ampholine pH 3–10, 5% β-mercaptoethanol) and 10 µl of glycerol, for a first separation by isoelectrofocusing (IEF). We used two different methods: (1) capillary gels containing 7% acrylamide, 3% NP 40, 0.3% ampholine pH 4–6, 0.3% ampholine pH 3.5–10 and 0.3% ampholine pH 5–8; IEF was run at 800 V, for 3 h. (2) Precoated commercial strips providing an immobilized pH gradient 4–7 (IPG), from Pharmacia (Righetti, 1990). In this case, focusing was run under the cover of paraffin oil and, in order to get good penetration of the sample into the gel, voltage was very progressively increased to 3.3 kV, then maintained overnight. Some samples were analyzed by both methods and identical results were obtained. For the second dimension, gels or strips were transferred to the top of a polyacrylamide slab gel (stacking gel: 3% polyacrylamide in 0.5 M Tris-HCl, sodium dodecylsulphate (SDS) 0.4%, pH 6.8; separating gel: 15% polyacrylamide in 1.5 M Tris-glycine, SDS 0.4%, pH 8.8) and covered with the running buffer (20 mM Tris, 192 mM glycine, 0.1% SDS). A sample of purified myosin from soleus muscle was run simultaneously. After staining, densitometry was integrated over the spot areas (Lecphor image analysis programme of Biocom, 91942 Les Ulis, France). The results are given as the percentage of phosphorylated (PMLC) and unphosphorylated forms of the total amount of the 20 kDa myosin light chains.

Drugs and solutions

The normal HEPES-Krebs solution contained in mM: Na⁺ 135.5, K⁺ 5.9, Mg²⁺ 1.2, Ca²⁺ 1.5, Cl⁻ 143.8, HEPES 11.6 and glucose 11.5. The pH was 7.3. K⁺ 140 mM solutions were obtained by replacing external Na⁺ by an equivalent amount of K⁺. Isoprenaline, ATP, indomethacin, ionomycin, 6-OHDA, ω-conotoxin and N^G-nitro-L-arginine were from Sigma. Methylene blue was obtained from Merck (Darmstadt, Germany) and adenosine 3':5'-cyclic monophosphorothioate (Rp diastereomer, Rp-cAMPS) from Biolog (Bremen, Germany). Fura-2/AM was from Molecular Probes (Eugene, Oregon, U.S.A.). All other reagents were of analytical grade. Drugs were dissolved in Krebs solution, except ionomycin which was dissolved in ethanol (stock solution 5 mM), and indomethacin, solubilized in 80% methanol containing 0.2 M NaOH (Griffith *et al.*, 1981) and used at the 1/1000 dilution of the stock solution.

Statistics

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. The results are presented as means ± s.e.mean, and *n* is the number of experiments.

Results

Factors affecting the ATP-induced relaxation

We previously showed that the mechanical counterpart of the P_{2Y} activation could be revealed on pre-contracted and depolarized preparations (15 min in 140 mM K⁺ solution): in these conditions, after a transient P_{2X}-related contraction, ATP produced a sustained and reversible relaxation induced by P_{2Y} activation (Boland *et al.*, 1992). The relaxant effect was maximal for a concentration of ATP of 1 mM. We describe here how this relaxation can be affected by various pharmacological interventions.

The relaxation induced by 1 mM ATP was unaffected by pre-incubation of the preparations, for 30 min, with 10 µM methylene blue, 10 µM indomethacin, or 100 µM N^G-nitro-L-arginine, agents known to inhibit the synthesis of cyclic GMP (Gruetter *et al.*, 1981), of prostaglandin (Vane, 1971), and of nitric oxide (Moore *et al.*, 1990; Toda *et al.*, 1990; Ishii *et al.*, 1990), respectively (Table 1). On the other hand, after 1 h incubation in the presence of 200 µM Rp-cAMPS, a cyclic AMP competitive inhibitor (Botelho *et al.*, 1988), the relaxant effect of ATP was significantly diminished (Table 1). The effect of Rp-cAMPS was further studied on the relaxation induced by 500 µM ATP. As illustrated in Figure 1, force contraction of VD maintained in 140 mM K⁺ stabilized at about one-fifth of its peak contraction, while [Ca²⁺]_i remained between 200 and 250 nM. In this condition, 500 µM ATP produced a rise of [Ca²⁺]_i followed by a return to its previous level after 3 min. This calcium increase was accompanied by a transient contraction followed within 30 s by a force decrease of about 35 ± 3.9% below the steady plateau level (*n* = 7). Thus, relaxation started while [Ca²⁺]_i was still well above its previous level (before ATP addition). This relaxation was maintained as long as ATP was applied, and was not associated with any further decrease of [Ca²⁺]_i. After 1 h incubation in presence of 200 µM Rp-cAMPS, the transient contraction induced by ATP was not significantly modified but the relaxant effect of ATP significantly diminished from 35 ± 3.9% to 10.7 ± 3.5% (*n* = 7) (Figure 1).

Isoprenaline (10 µM) mimicked the effect of ATP 500 µM as it induced a relaxation of 35 ± 5% (*n* = 7) after 30 s, which was maintained as long as isoprenaline was applied (Figure 1), without affecting the level of [Ca²⁺]_i. This β-agonist is well known to produce an increase of [cyclic AMP] in smooth muscle (Scheid *et al.*, 1979). As expected, we found that the relaxation induced by isoprenaline was completely abolished by pre-incubation with 200 µM Rp-cAMPS (*n* = 5).

Changes of cyclic AMP concentration

The similarity of the relaxant effects of ATP and isoprenaline on force and the sensitivity of these effects to the Rp-cAMPS

Table 1 The effect of various agents on ATP-induced relaxation of the vas deferens

Pre-incubation conditions	ATP-(1 mM)-induced relaxation†
Control (K ⁺ 140 mM)	- 56 ± 6%, <i>n</i> = 7
Indomethacin 10 µM	- 58 ± 7%, <i>n</i> = 4
Methylene blue 10 µM	- 50 ± 9%, <i>n</i> = 3
N ^G -nitro-L-arginine 100 µM	- 51 ± 2%, <i>n</i> = 4
Rp-cAMPS 200 µM	- 19 ± 3%, <i>n</i> = 4*

†Drop in tension, expressed as % of the steady tone level maintained by prolonged K⁺-depolarization.

**P* < 0.01: *t* test.

Figure 1 (a) Shows the changes in $[Ca^{2+}]_i$ (upper trace) and force (lower trace) in the vas deferens in response to superfusion with K^+ 140 mM, ATP 500 μ M and isoprenaline 10 μ M (Iso). (b) Presents the same experiment after 1 h incubation with Rp-cAMPS. Horizontal bars indicate the timing of the superfusions.

inhibitor suggested the participation of cyclic AMP in the relaxation of the depolarized preparation. Therefore, we measured [cyclic AMP] directly in muscle extracts. In control, resting conditions, [cyclic AMP] was 636 ± 18 fmol mg^{-1} of wet muscle ($n = 16$). A similar basal level was reported by others (Diamond & Janis, 1978; Heller *et al.*, 1989). After 5 min in 100 μ M ATP, [cyclic AMP] rose to 760 ± 46 fmol mg^{-1} ($n = 16$), and to 939 ± 113 fmol mg^{-1} ($n = 5$), in 1000 μ M ATP. As a point of comparison, isoprenaline (10 μ M, 5 min) produced an increase of [cyclic AMP] to 937 ± 87 fmol mg^{-1} ($n = 6$). All these increases were significantly higher than the basal level.

Myosin light chain phosphorylation

At rest, the relative amount of phosphorylation of the 20 kDa myosin light chain (PMLC) amounted to $5.4 \pm 2.9\%$ ($n = 8$). At the initial peak of contraction during depolarization with 140 mM K^+ , PMLC reached $31.8 \pm 4.1\%$ ($n = 7$); after 25 min, PMLC had declined to $16.85 \pm 1.4\%$ ($n = 19$, control situation). When ATP (1000 μ M) was added to the pre-contracted VD, after 15 min in 140 mM K^+ , and the muscle frozen 10 min later (total duration in 140 mM K^+ : 25 min), PMLC had decreased to $12.75 \pm 1.2\%$ ($n = 20$). The difference compared with the control situation was significant. Thus in pre-contracted and depolarized VD, ATP produced a force relaxation and a myosin LC dephosphorylation of about the same relative amplitude.

Test of the two antagonistic pathways hypothesis

We previously suggested that calcium-force dissociation observed in resting VD with ATP (Boland *et al.*, 1992) might be explained by an overlap of contracting and relaxing influences; the latter was shown, here, to be related to cyclic AMP production. Two experiments were designed to test this hypothesis.

(i) If the ATP-induced synthesis of cyclic AMP is responsible for the calcium-force dissociation in VD, then incubation of resting VD with Rp-cAMPS would be expected to improve the calcium-force coupling, and thereby to increase the amplitude of the contraction produced by ATP. Stimulation with 100 μ M ATP, which induces an obvious calcium-force dissociation (Boland *et al.*, 1992), significantly elevated [cyclic AMP] (see above) and induced a contraction of only $3 \pm 1\%$ ($n = 9$) of the maximal force developed in response to 140 mM K^+ depolarization. This force response was not increased by pre-incubation with 200 μ M Rp-cAMPS ($2.25 \pm 0.75\%$, $n = 4$).

(ii) The simple model of overlapping increases of $[Ca^{2+}]_i$ and of [cyclic AMP]_i can be mimicked by combining K^+ depolarization (Boland *et al.*, 1992) with the presence of isoprenaline (see above). We found that K^+ -induced contraction was not significantly affected by concomitant addition of 10 μ M isoprenaline ($97 \pm 6.5\%$ of the K^+ reference peak, $n = 4$). However, after a pre-incubation with 20 μ M isoprenaline for 2.5 min, the peak contraction induced by K^+ depolarization attained $76 \pm 2\%$ ($n = 7$) of the reference value. Obviously, an antagonistic effect of Ca^{2+} and cyclic AMP on the force response can be detected in the latter condition, but it is not of the required magnitude to explain the very weak force observed in the presence of ATP.

Discussion

We previously showed that ATP can induce a transient contraction followed by a sustained relaxation in depolarized preparations and that the latter effect probably involved P_{2Y} -purinoceptors (Boland *et al.*, 1992). In this paper, different intracellular mechanisms able to induce smooth muscle relaxation were investigated.

ATP-induced relaxation could be mediated by a decrease of $[Ca^{2+}]_i$ but the finding that relaxation started during the peak of calcium and was maintained at an elevated and steady $[Ca^{2+}]_i$ (around 250 nM, see Figure 1) does not support this interpretation. ATP can also induce opening of K^+ -channels and hence hyperpolarization, in guinea-pig taenia coli (Tomita & Watanabe, 1973) and in ileum circular layers (Crist *et al.*, 1992). Obviously, membrane hyperpolarization is not involved here as K^+ -depolarized VD showed relaxation in response to ATP. Prostaglandin synthesis and ATP-induced relaxation are linked in guinea-pig taenia coli (Brown & Burnstock, 1981); but, as relaxation of VD was unaffected by indomethacin, this pathway seems unlikely. In vascular smooth muscle, relaxation can also be mediated through the P_{2Y} -induced release of nitric oxide by endothelium, producing an activation of the smooth muscle guanylyl cyclase. Recently, NO has also been reported as a neurotransmitter in non vascular (Li & Rand, 1990; Boeckxstaens *et al.*, 1991; Lefebvre *et al.*, 1992) and vascular smooth muscle (Toda & Okamura, 1992). Muscle tissue could even directly generate a muscle-derived relaxing factor (MDRF) similar to NO (Wood *et al.*, 1990). In our preparation, we found that relaxation was unaffected by inhibitors of NO production. Stimulation of cyclic GMP synthesis, independent of the NO pathway, can also be excluded, as relaxation was insensitive to methylene blue.

P_{2Y} -purinoceptors are linked to a G protein (Kennedy, 1990) and could activate cytosolic adenylyl cyclase. Three observations support the proposal that cyclic AMP is mediating the ATP-induced relaxation in the K^+ -pre-

contracted VD. (1) Relaxation is partially inhibited by Rp-cAMPS, a competitive antagonist of cyclic AMP. (2) We observed some similarities of the responses to ATP and to isoprenaline, a β -adrenoceptor agonist, well known to induce relaxation by the synthesis of cyclic AMP (Scheid *et al.*, 1979): in K^+ -depolarized preparations, both isoprenaline and ATP caused sustained relaxations of comparable amplitudes, which occurred in spite of an elevated $[Ca^{2+}]_i$, and were significantly inhibited by Rp-cAMPS. (3) In physiological conditions, direct measurements showed that externally applied ATP produced a rise of the cyclic AMP concentration similar to that produced by isoprenaline.

ATP-induced relaxation of depolarized VD was correlated with a reduction of the level of PMLC. Most likely this reflected the inhibition of the myosin light chain kinase (MLCK) when it is phosphorylated by a cyclic AMP-dependent protein kinase A (Adelstein *et al.*, 1978; Kerrick & Hoar, 1981; Nishimura & van Bremen, 1989; Ozaki *et al.*, 1992).

On the basis of the rank order of potency of different ATP analogues, of the absence of tachyphylaxis and of the action of selective antagonists, it was proposed that ATP-induced relaxation implies a P_{2Y} -like purinoceptor (Boland *et al.*, 1992). Our results suggest that this effect is linked to the activation of adenylyl cyclase. This pathway is well documented for P_1 -purinoceptors (A_2 subclass; for review, see Kennedy, 1990) but has not been reported for P_{2Y} -purinoceptors. However, the possibility that the receptor could be another sub-type of P_2 -purinoceptor (P_{2Z} , P_{2T} or unknown) cannot be definitely excluded (for review, see Olson & Pearson, 1990).

Together, these and our previous results indicate that externally applied ATP gives rise to two intracellular messengers, Ca^{2+} and cyclic AMP. The first one, in combination with calmodulin, would lead to activation of MLCK, while the second, through the cyclic AMP-protein kinase A cascade, to the inhibition of MLCK (for review, see Rasmussen & Rasmussen, 1990). As these two antagonistic influences overlap somewhat in time, the force response would reflect the net result of these influences at the level of MLCK. This could be the basis of the calcium-force dissociation we previously reported. A similar model of two antagonistic influences has been proposed by Watanabe and co-workers (1992) to explain the calcium-force dissociation observed in caffeine-treated aorta. However, in the case of the VD, it seems that the simultaneous elevation of $[Ca^{2+}]_i$ and [cyclic AMP] would not be sufficient to explain fully the calcium-force dissociation. Indeed, when the cyclic AMP pathway was inhibited, the weak ATP-induced force was not potentiated, contrary to expectations. Moreover, in experiments meant to mimic the simultaneous increases of $[Ca^{2+}]_i$ and [cyclic AMP] (stimulation with K^+ and isoprenaline), the observed antagonistic influence of the two second messengers on the force response was much too small to explain the calcium-force dissociation induced by ATP.

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