ELSEVIER

European Journal of Pharmacology 436 (2002) 135–143

ejp

www.elsevier.com/locate/ejphar

Nucleotide-evoked relaxation of rat vas deferens: possible mechanisms

R. Bültmann *, W. Klebroff, K. Starke

Pharmakologisches Institut, Albertstrasse 25, D-79104 Freiburg i.Br., Germany

Received 2 August 2001; received in revised form 14 December 2001; accepted 21 December 2001

Abstract

ATP causes relaxation of the K^+-contracted rat vas deferens. Possible sites of action were investigated. ATP and adenosine relaxed the vas deferens precontracted with 80 mM K^+; EC_{50} values and maximal relaxations averaged, respectively, 760 μM and 56% for ATP and 74 μM and 30% for adenosine. The adenosine P1 receptor antagonist 8-(*para*-sulfophenyl)theophylline (8-SPT) reduced relaxations caused by adenosine and low concentrations of ATP, as did the Rp-diastereomer of adenosine 3′,5′-cyclic phosphorothioate (Rp-cAMPS), an inhibitor of protein kinase A. The phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) augmented responses to adenosine and low concentrations of ATP. α,β-Methylene ADP, an inhibitor of 5′-nucleotidase, reduced relaxations caused by ATP to a similar extent as did 8-SPT. In the presence of an almost saturating concentration of adenosine, ATP caused further relaxation. Conversely, in the presence of ATP, adenosine had little effect. Like ATP, UTP and other nucleoside triphosphates relaxed the vas deferens. The P2 receptor antagonists reactive blue 2, acid blue 25 and 4,4′-diisothiocyanotostilbene-2,2′-disulphonate (DIDS) attenuated the relaxation caused by ATP; suramin, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonate (PPADS), Evans blue, trypan blue, reactive red 2 and brilliant blue G had no effect. Three non-selective inhibitors of protein kinases, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), staurosporine and (8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-carboxy-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a*,*g*]cycloocta[*cde*]trinden-1-one (K-252b), markedly reduced the relaxation caused by ATP. The results indicate that adenosine, derived from enzymatic dephosphorylation, contributes to the relaxant effect of ATP, presumably by activation of a smooth muscle adenosine receptor linked to the accumulation of cAMP and activation of protein kinase A. Yet, the main part of the response to ATP is mediated by a site distinct from the adenosine receptor. The pharmacological properties of this site differ from known P2 receptor subtypes. Possibly, the nucleotide-evoked relaxation is due to a phosphoryl transfer catalyzed by an *ecto*-protein kinase.

Keywords: Vas deferens, rat; ATP; Adenosine; Smooth muscle relaxation; P2 receptor; Adenosine A2 receptor; *Ecto*-protein kinase

1. Introduction

The *contraction* of the longitudinal smooth muscle of the vas deferens in response to extracellular nucleotides is a long known phenomenon (e.g., Clanachan et al., 1977; Fedan et al., 1982; Von Kügelgen et al., 1990; Bailey and Hourani, 1994; Khakh et al., 1995; Bültmann et al., 1999a). More recently, ATP has been shown to cause *relaxation* of mouse and rat vas deferens after the tone of the preparation had been raised by high potassium (Boland et al., 1992; Gailly et al., 1993; Bültmann and Starke, 2001). This second type of response is of particular interest, because endogenous ATP, released from postjunctional sites upon α_1-adrenoceptor stimulation, also causes relaxation of the tissue (Bültmann and Starke, 2001).

Based on experiments in the vas deferens of the *mouse*, Boland et al. (1992) suggested that ATP and its analogue 2-methylthio ATP (MeSATP) cause relaxation through a smooth muscle P_{2Y} purinoceptor stimulating the accumulation of cAMP (Boland et al., 1992; Gailly et al., 1993). However, this interpretation seems questionable, since ATP and MeSATP were about equieffective, and relatively high concentrations (≥ 10 μM) of both agonists were required to elicit relaxation. Moreover, the P2 receptor antagonist reactive blue 2, when tested at a concentration of 200 μM, only slightly reduced the response to ATP (Boland et al., 1992). At smooth muscle P_{2Y} purinoceptors in other tissues, MeSATP is considerably more potent than ATP, causing relaxation at nanomolar concentrations (reviewed by Burnstock and Kennedy, 1985; Kennedy and Leff, 1995), and

* Corresponding author. Tel.: +49-761-203-5313; fax: +49-761-203-5318.

E-mail address: bueltman@pironet.de (R. Bültmann).

0014-2999/02/$ - see front matter
PII: S0014-2999(01)01620-X

reactive blue 2 is a potent antagonist (e.g., Crema et al., 1983; Burnstock et al., 1986; Bültmann et al., 1996). The $P2Y_1$ receptor, which is thought to represent the molecular correlate of the 'classical' smooth muscle P_{2Y} purinoceptor, is also activated by nanomolar concentrations of MeSATP and blocked by reactive blue 2 (Webb et al., 1993; Filtz et al., 1994; Tokuyama et al., 1995; see Harden et al., 1998 and Von Kügelgen and Wetter, 2000 for the nomenclature of P2Y receptors).

Similarly, in the vas deferens of the *rat*, ATP and MeSATP were about equieffective in causing relaxation (Bültmann and Starke, 2001); but adenosine 5′ -*O*-(2-thiodiphosphate) (ADPβS), a prototype $P2Y_1$ receptor agonist (Harden et al., 1998), had no effect. Although the relaxant effect of ATP was attenuated by moderate concentrations of reactive blue 2 (10 μM and above), it remained unchanged in the presence of suramin and acid blue 129, which block relaxation-mediating P2 receptors in other tissues (e.g., Bültmann et al., 1996; Johnson et al., 1996; Tuluc et al., 1998). Based on these findings, it has been suggested that the site of action of the nucleotides in rat vas deferens is not a typical smooth muscle P_{2Y} purinoceptor (Bültmann and Starke, 2001).

The possible physiological significance of the mechanism (see Bültmann and Starke, 2001) prompted us to investigate the nucleotide-evoked relaxation in greater detail. The vas deferens of the rat is known to possess adenosine A2 receptors mediating smooth muscle relaxation (Huidobro-Toro and Parada, 1989; Hourani et al., 1993; Martin and May, 1994; Brownhill et al., 1996) and exogenous ATP is rapidly broken down by *ecto*-nucleotidases present in the tissue (Bültmann et al., 1995; Khakh et al., 1995), yielding, among other metabolites, also small amounts of adenosine (Tennant et al., 1999). Despite our initial failure to detect a contribution of adenosine to the relaxant effect of ATP (Bültmann and Starke, 2001), we also re-addressed this possibility.

2. Materials and methods

2.1. General

Male Wistar rats (220–320 g) were killed by decapitation and the vasa deferentia removed and cleaned of adherent tissue. Prostatic thirds were suspended vertically in a 6.1-ml organ bath. The lower end was fixed and the upper end attached to an isometric force transducer (K30, Hugo Sachs Elektronik, Hugstetten, FRG) under an initial tension of 9.8 mN (Graphtec thermal pen recorder, Ettlingen, FRG). Unless stated otherwise, the bath fluid was replaced every 15 min. The medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 0.9, $NaHCO_3$ 25, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. It was saturated with 95% O_2:5% CO_2 and kept at 37 °C. Tissues relaxed to about 3 mN during a 60-min equilibration period. This final resting tension remained constant for the remainder of the experiments.

In order to induce a tonic contraction of the vas deferens, the concentration of K^+ in the medium was raised to 80 mM by isomolar replacement of NaCl with KCl (cf. Boland et al., 1992; Bültmann and Starke, 2001). The first two additions of K^+, 60 and 120 min after beginning of the experiment, did not yield a stable plateau. Only the following one (180 min) or two additions (180 and 210 min after beginning of the experiment) yielded a stable tonic contraction and served to determine agonist-induced relaxation. Agonists (adenosine or nucleotides) were administered either at a single concentration or in a cumulative fashion during the plateau of the K^+ response, i.e. from about 10 min after the addition of K^+ onwards. Unless stated otherwise, agonists were washed out together with K^+ when the relaxation elicited by the highest concentration was maximal. It took about 5 to 15 min to determine one concentration–relaxation curve. Relaxations were measured at their maximum and expressed as a percentage of the respective K^+ contraction. Further experimental details are given in the Results.

Agonist EC_{50} values and the apparent antagonist K_d value of 8-(*para*-sulfophenyl)theophylline (8-SPT) against adenosine were determined as follows. Logistic curves were fitted to the weighted mean relaxation values by using equation no. 25 of Waud (1976) and nonlinear regression. The calculation yielded the maximal effect and the EC_{50}, i.e. the concentration producing 50% of the maximum of that curve. The apparent antagonist K_d value was derived from the shift of the adenosine concentration–response curve in the presence of 8-SPT, read at the level of the EC_{50} and corrected for the mean shift occurring in solvent controls. The apparent K_d value was calculated using equation no. 4 of Furchgott (1972).

2.2. Statistics

Data are expressed as either the arithmetic mean ± S.E.M. or, in the case of EC_{50} values and maximal effects, the S.E. as defined by Waud (1976). Means were tested for a significant difference by the Mann–Whitney test, with Bonferroni correction if applicable. $P < 0.05$ was taken as the limit of statistical significance.

2.3. Materials

Special chemicals were acid blue 25 (Aldrich, Steinheim, Germany; purified by column chromatography; Tuluc et al., 1998); suramin (Bayer, Wuppertal, Germany); 2-methylthio ATP tetrasodium (MeSATP), 8-(*para*-sulfophenyl)theophylline (8-SPT), reactive blue 2 (Biotrend, Köln, Germany); adenosine 3′ ,5′ -cyclic phosphorothioate triethylammonium (Rp-diastereomer; Rp-cAMPS), 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724), (8*R**,9*S**,11*S**)-(−)-9-hydroxy-9-carboxy-8-methyl-2,3,9,10-tetrahydro-

8,11-epoxy-1*H*,8*H*,11*H*-2,7b,11a-triazadibenzo[*a*,*g*]cyclooctа[*cde*]trinden-1-one (K-252b; Calbiochem, Bad Soden, Germany); pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid disodium (PPADS; Cookson, Southampton, UK); adenosine, adenosine 5′-*O*-(2-thiodiphosphate) trilithium (ADPβS), adenosine 5′-*O*-(2-thiotriphosphate) tetralithium (ATPγS), adenosine 5′-triphosphate disodium (ATP), brilliant blue G, cytidine 5′-triphosphate disodium (CTP), 4,4′-diisothiocyanotostilbene-2,2′-disulphonic acid disodium (DIDS), Evans blue, guanosine 5′-triphosphate tetralithium (GTP), inosine 5′-triphosphate trisodium (ITP), 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), α,β-methylene ADP disodium (α,β-MeADP), α,β-methylene ATP dilithium (α,β-MeATP), β,γ-methylene ATP disodium (β,γ-MeATP), nifedipine, staurosporine, trypan blue, uridine 5′-triphosphate trisodium (UTP) (Sigma, Deisenhofen, Germany); reactive red 2 (synthesized in our laboratory as previously described; Bültmann and Starke, 1995). K-252b, Ro 20-1724 and staurosporine were dissolved in dimethylsulphoxide (DMSO; final concentration below 0.1%); nifedipine was dissolved in ethanol (final concentration below 0.1%); all other compounds were dissolved in distilled water. Solutions of drugs were added to the organ bath in aliquots not exceeding 100 μl.

3. Results

Increasing the concentration of K^+ in the bath fluid from 5.7 to 80 mM caused a biphasic contraction of the rat vas deferens (Fig. 1; cf. Triggle et al., 1979; Hay and Wadsworth, 1982); an initial transient phase was followed by a secondary tonic contraction (9.9 ± 0.2 mN; $n = 267$). The

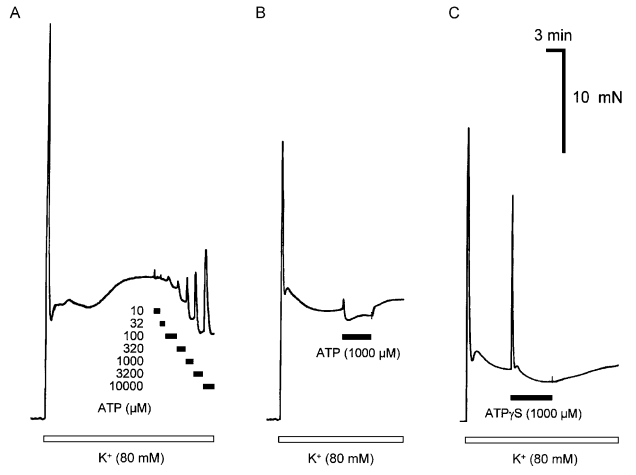

Fig. 1. Contraction of the rat vas deferens by K^+ and relaxation by ATP and ATPγS: original recordings. K^+ (final concentration 80 mM) was added to the medium, and ATP was administered during the plateau of the ensuing contraction, either at cumulatively increasing concentrations (A) or at a single concentration (B); ATPγS was administered at a single concentration (C). Representative tracings from three to seven experiments. Note different time course of the response to ATP (B) and ATPγS (C).

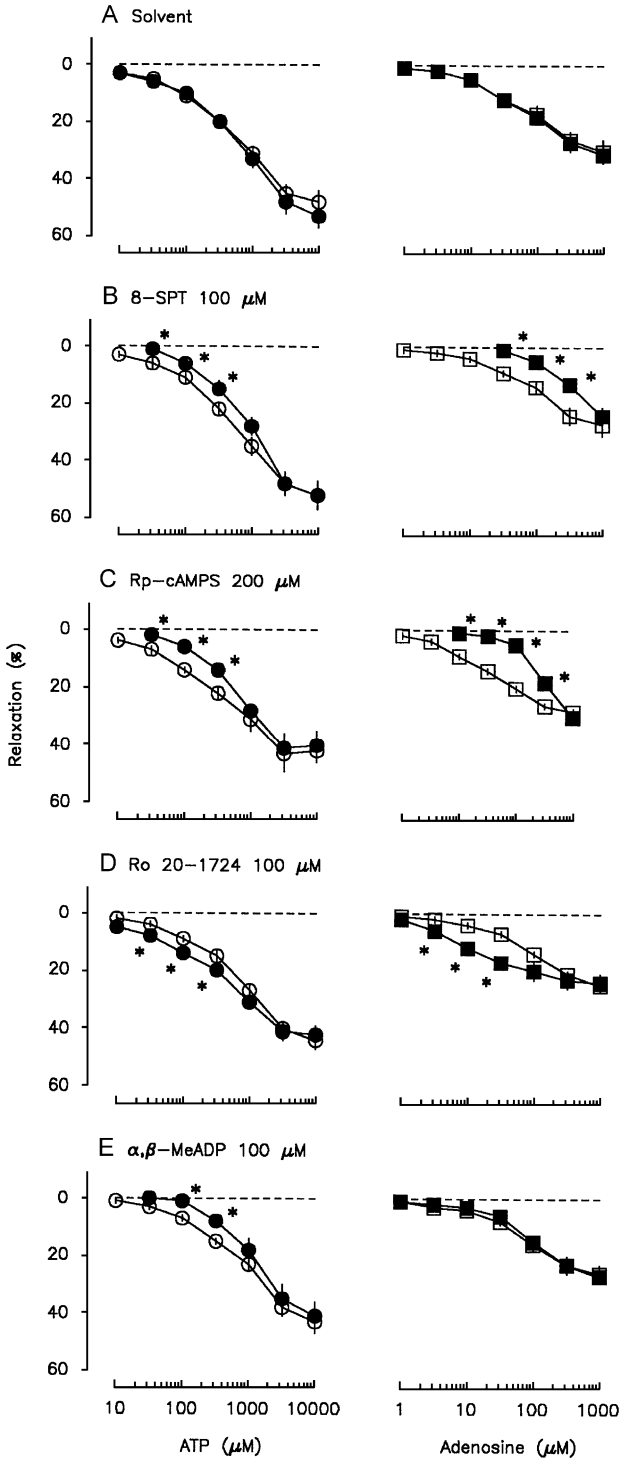

Fig. 2. Relaxation of the K^+-contracted rat vas deferens by ATP and adenosine: effect of inhibitors. K^+ (final concentration 80 mM) was added to the medium twice, interval 60 min. ATP (circles) or adenosine (squares) was administered in a cumulative fashion during the plateau of each response to K^+. Solvent (A), 8-SPT (100 μM; B), Rp-cAMPS (200 μM; C), Ro 20-1724 (100 μM; D) or α,β-MeADP (100 μM; E) was added immediately after the first ATP or adenosine concentration–relaxation curve, i.e. about 50 min before the second curve. *Abscissae*, agonist concentration. *Ordinates* show relaxation in first curves (open symbols) and second curves in the presence of solvent or inhibitor (filled symbols), as a percentage of the respective response to K^+. Means ± S.E.M. from three to seven experiments. * denotes a significant difference ($P < 0.05$) from solvent.

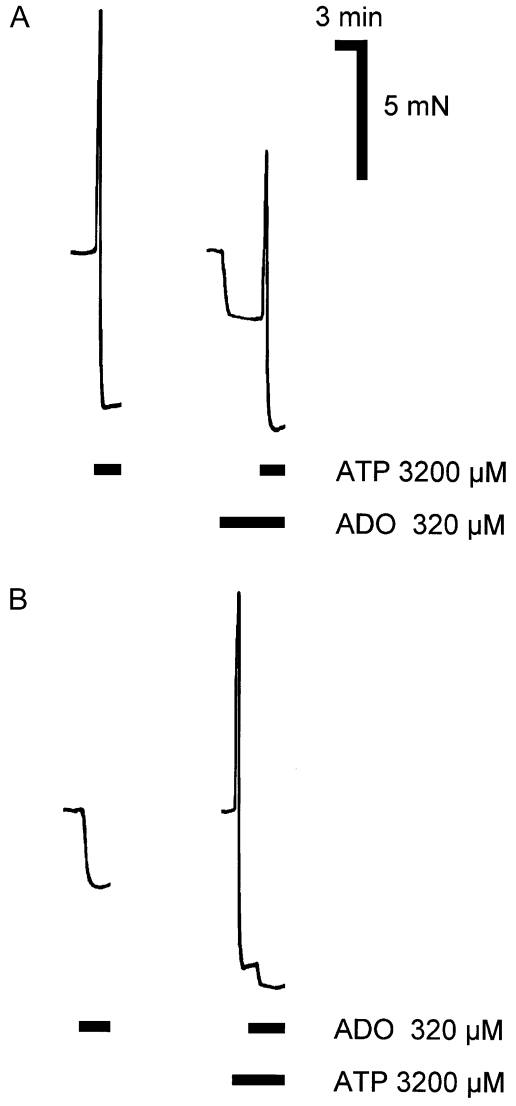

Fig. 3. Relaxation of the K^+-contracted rat vas deferens by ATP and adenosine: combined administration. K^+ (final concentration 80 mM) was added to the medium twice at 30-min intervals. Each single tracing starts with the plateau of the K^+-evoked contraction. ATP (3200 μM) and adenosine (ADO; 320 μM) were administered as indicated. Representative tracings from four or five experiments.

latter remained stable while the concentration of K^+ remained elevated (except during the first two — discarded — exposures to 80 mM K^+; see Methods).

ATP (10–10000 μM), when added during the plateau of the response to K^+, caused a fast and transient contraction, followed by a slower and sustained relaxation (Fig. 1A,B; cf. Bültmann and Starke, 2001; throughout the present study, contractions evoked by the nucleotides were not evaluated). The response was rapidly reversible upon washout of the nucleotide (Fig. 1B). Relaxations increased progressively with increasing concentrations of ATP (Fig. 1A and open circles in Fig. 2; EC_{50} 760 ± 120 μM; maximal relaxation 56 ± 2%; all first concentration–relaxation curves pooled; $n = 107$). Adenosine (1–1000 μM) caused no contraction, but only rapid and sustained relaxation (Fig. 3 below and open squares in Fig. 2; EC_{50} 74 ± 8 μM; maximal relaxation 30 ± 1%; all first concentration–relaxation curves pooled; $n = 23$).

When two concentration–relaxation curves of ATP or adenosine were determined in a single preparation, the second curve, after addition of solvent (filled symbols in Fig. 2A), was similar to the first (open symbols in Fig. 2A). The adenosine P1 receptor antagonist 8-SPT (100 μM) shifted the concentration–relaxation curve of adenosine to the right, yielding an apparent antagonist K_d value of 47 μM, and slightly but significantly attenuated relaxations caused by lower concentrations of ATP (Fig. 2B). Likewise, Rp-cAMPS (200 μM), an antagonist of cAMP and, hence, an inhibitor of protein kinase A, attenuated relaxations caused by adenosine and low concentrations of ATP (Fig. 2C). On the other hand, the phosphodiesterase inhibitor Ro 20-1724 (100 μM) shifted the concentration–relaxation curve of adenosine to the left and augmented relaxations caused by lower concentrations of ATP (Fig. 2D). Finally, α,β-MeADP (100 μM), an inhibitor of 5′ -nucleotidase, did not alter the concentration–relaxation curve of adenosine but attenuated relaxations caused by ATP to a similar extent as did 8-SPT and Rp-cAMPS (Fig. 2E). The combined administration of 8-SPT (100 μM) plus either Rp-cAMPS (200 μM), Ro 20-1724 (100 μM) or α,β-MeADP (100 μM) did not change the concentration–relaxation curve of ATP beyond the change caused by 8-SPT alone ($n = 3$ to 4).

Effects were tested of the successive administration of almost saturating concentrations of ATP and adenosine. ATP (3200 μM) or adenosine (320 μM), when added during the plateau of the contraction elicited by potassium, caused relaxation by 51 ± 6% and 26 ± 4%, respectively (Fig. 3; first addition). When added in the continued presence of adenosine (320 μM), ATP (3200 μM) caused further relaxation (Fig. 3A). In the presence of ATP (3200 μM), on the

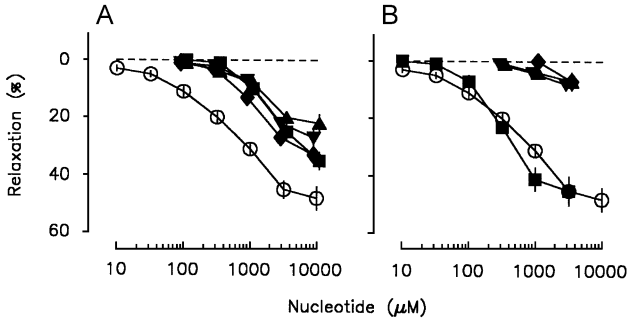

Fig. 4. Relaxation of the K^+-contracted rat vas deferens by various nucleotides. K^+ (final concentration 80 mM) was added to the medium once. ATP (○; A and B), CTP (♦; A), GTP (■; A), ITP (▲; A), UTP (▼; A), MeSATP (■; B), α,β-MeATP (▲; B), β,γ-MeATP (▼; B) or ADPβS (♦; B) was administered in a cumulative fashion during the plateau of the response to K^+. *Abscissae*, agonist concentration. *Ordinates* show relaxation, as a percentage of the respective response to K^+. Means ± S.E.M. from three to seven experiments.

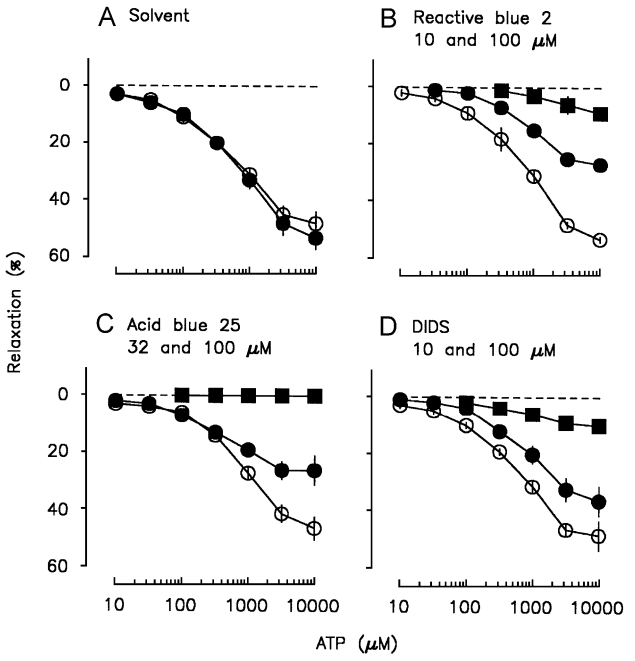

Fig. 5. Relaxation of the K^+-contracted rat vas deferens by ATP: effect of P2 receptor antagonists. K^+ (final concentration 80 mM) was added to the medium twice, interval 60 min. ATP was administered in a cumulative fashion during the plateau of each response to K^+. Solvent (A), reactive blue 2 (10 μM ●; 100 μM ■; B), acid blue 25 (32 μM ●; 100 μM ■; C) or DIDS (10 μM ●; 100 μM ■; D) was added immediately after the first ATP concentration–relaxation curve, i.e. about 50 min before the second curve. *Abscissae*, ATP concentration. *Ordinates* show relaxation in first curves (○) and second curves in the presence of solvent or inhibitor (filled symbols), as a percentage of the respective response to K^+. For the sake of clarity, first curves are shown for the lower inhibitor concentration only. First curves from experiments using the higher concentration were almost identical. Means ± S.E.M. from three to seven experiments.

other hand, adenosine (320 μM) had little effect (Fig. 3B). Irrespective of the order of application, the overall response caused by ATP plus adenosine amounted to 59 ± 3% relaxation ($n = 9$; all experiments pooled), not significantly different from the relaxation caused by ATP alone.

Like ATP, the naturally occurring nucleoside triphosphates UTP, CTP, GTP and ITP caused relaxation of the vas deferens when added during the plateau of the contraction elicited by K^+; ATP was the most potent agonist, the other nucleotides were less and about equipotent (Fig. 4A). Among analogues of ATP, only MeSATP and ATPγS caused marked relaxation. MeSATP (EC_{50} 329 ± 40 μM; maximal relaxation 48 ± 4%; $n = 4$) and ATP were about equipotent (Fig. 4B). The response to ATPγS (not in Fig. 4) developed slowly and was slowly reversible upon washout (Fig. 1C). Therefore, the amplitude of the ATPγS relaxation could not be reliably evaluated, but the magnitude of the responses to ATP (1000 μM) and ATPγS (1000 μM; Fig. 1B,C) indicate that the two nucleotides were about equieffective. α,β-MeATP, β,γ-MeATP and ADPβS caused less than 10% relaxation at the highest concentration tested (3200 μM; Fig. 4B).

P2 receptors are one possible site of action of extracellular nucleotides. The P2 receptor antagonists reactive blue 2 (10 and 100 μM), acid blue 25 (32 μM) and DIDS (10 and 100 μM) reduced the maximum of the concentration–response curve of ATP; acid blue 25 (100 μM) abolished the relaxation over the entire range of ATP concentrations tested (Fig. 5). In contrast, suramin (100 μM), PPADS (100 μM), Evans blue (100 μM), trypan blue (100 μM), reactive red 2 (10 μM) and brilliant blue G (100 μM) did not alter the concentration–relaxation curve of ATP ($n = 3$ to 5; not shown).

Apart from being agonists at P2 receptors, extracellular nucleotides also serve as substrates for *ecto*-protein kinases (for review see Ehrlich et al., 1990; Redegeld et al., 1999). Since *ecto*-protein phosphorylation by ATP has been demonstrated in guinea-pig vas deferens (Lamport-Vrana et al., 1991), we also addressed the possibility that the relaxation of the vas deferens following addition of ATP was due to the activity of *ecto*-protein kinases. Three nonselective inhibitors directed against the catalytic domain of several protein kinases (see Rüegg and Burgess, 1989), H-7 (10 μM), staurosporine (0.1 μM) and K-252b (5 μM) markedly attenuated the relaxation caused by ATP (Fig. 6).

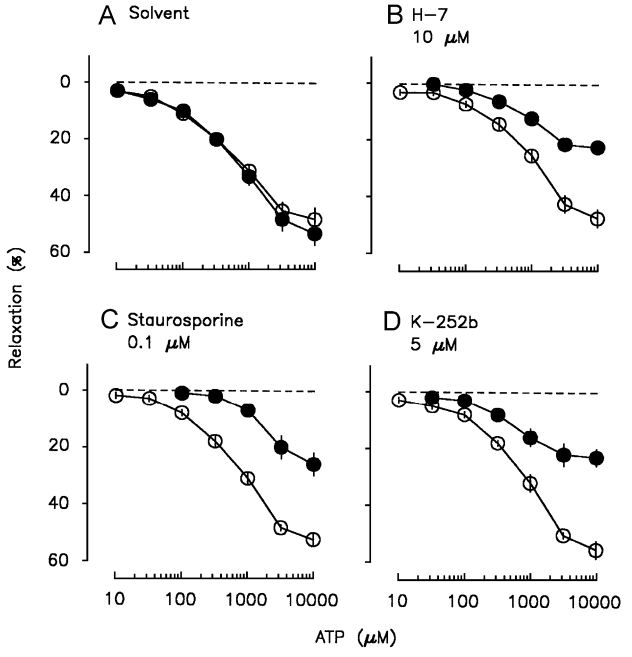

Fig. 6. Relaxation of the K^+-contracted rat vas deferens by ATP: effect of protein kinase inhibitors. K^+ (final concentration 80 mM) was added to the medium twice, interval 60 min. ATP was administered in a cumulative fashion during the plateau of each response to K^+. Solvent (A), H-7 (10 μM ●; B) or staurosporine (0.1 μM ●; C) was added immediately after the first ATP concentration–relaxation curve, i.e. about 50 min before the second curve. K-252b (5 μM ●; D) was added 10 min before the second curve. *Abscissae*, ATP concentration. *Ordinates* show relaxation in first curves (○) and second curves in the presence of solvent or inhibitor (filled symbols), as a percentage of the respective response to K^+. Means ± S.E.M. from three to seven experiments.

Ro 20-1724 (100 μM), reactive blue 2 (100 μM), acid blue (32 and 100 μM), H-7 (10 μM), staurosporine (0.1 μM) and K-252b (5 μM) reduced the plateau of the contraction elicited by potassium (by 15 to 67%); all other compounds caused no change. In order to rule out the possibility that an apparent inhibition of the relaxation caused by ATP had been due to a reduction of the response to K^+, effects of the Ca^{2+} channel blocker nifedipine were investigated. Nifedipine (0.01 and 0.032 μM) reduced the plateau of the contraction elicited by potassium by 40% and 73%, respectively, but did not alter the concentration–relaxation curve of ATP ($n=4$ each; not shown).

4. Discussion

Our results confirm the principal observation of Boland et al. (1992) and Bültmann and Starke (2001): extracellular nucleotides cause relaxation of the vas deferens after the tone of the preparation has been raised by high K^+. Yet, there are differences between mouse (Boland et al., 1992) and rat vas deferens (Bültmann and Starke, 2001; present study) with respect to the site of action of the nucleotides.

4.1. Adenosine

In the mouse vas deferens, adenosine was less effective than ATP in eliciting relaxation, and the adenosine receptor antagonist 8-phenyltheophylline did not alter the concentration–relaxation curve of ATP. Both findings argued against a possible contribution of adenosine to the relaxant effect of ATP (Boland et al., 1992). In rat vas deferens, in contrast, adenosine was more potent than ATP, and several observations indicate that adenosine, derived from the breakdown of added ATP, contributes to the relaxant effect of the nucleotide.

The relaxation of the rat vas deferens caused by adenosine was antagonized by 8-SPT with an apparent antagonist K_d value of 47 μM (Fig. 2B); it was attenuated by Rp-cAMPS, an inhibitor of protein kinase A activation (Fig. 2C), and was enhanced by the phosphodiesterase inhibitor Ro 20-1724 (Fig. 2D). These findings are compatible with the idea that adenosine activates a smooth muscle adenosine receptor, presumably A2 (Bruns et al., 1986; see Introduction), leading to an accumulation of cAMP and activation of protein kinase A.

The relaxation effects of ATP and adenosine were less than additive (Fig. 3), indicating that both agonists share a common mechanism of action. In support of this concept, the relaxation caused by lower concentrations of ATP was affected by 8-SPT, Rp-cAMPS and Ro 20-1724 in a manner similar to the concentration–response curve of adenosine (Fig. 2B–D), demonstrating a small—and previously undetected (Bültmann and Starke, 2001)—adenosine component in the effect of ATP. The 5′-nucleotidase inhibitor α,β-MeADP (100 μM) attenuated relaxations caused by ATP to a similar extent as did 8-SPT (100 μM; Fig. 2B,E), and combined administration of the two compounds caused no further inhibition. The adenosine-like effect of ATP is, hence, not due to a direct activation of the adenosine receptor by ATP but depends on prior degradation of the nucleotide to adenosine (see Introduction).

Notably, any changes were small and the effect of high concentrations of ATP remained unchanged in the presence of 8-SPT, Rp-cAMPS, Ro 20-1724 and α,β-MeADP (Fig. 2). Moreover, in the presence of an almost saturating concentration of adenosine, ATP caused further relaxation (Fig. 3A). Therefore, the main part of the effect of ATP is mediated by a site distinct from the adenosine receptor. Two possibilities were tested.

4.2. P2 receptors

In the mouse vas deferens, the relaxation caused by ATP and MeSATP has been suggested to be mediated by a P2 receptor, specifically a P_{2Y} purinoceptor (Boland et al., 1992). This concept, though at variance with the proposed pharmacology of smooth muscle P_{2Y} purinoceptors (see Introduction), has gained some support from the recent cloning of a P2 receptor ($P2Y_{11}$) that stimulates adenylate cyclase and is selectively activated by ATP and MeSATP (Communi et al., 1997). MeSATP and ATP are also the most potent agonists in rat vas deferens (Fig. 4). Yet, several observations indicate that the site of action of the nucleotides in rat vas deferens is neither a 'classical' P_{2Y} purinoceptor (i.e. $P2Y_1$; Harden et al., 1998; Von Kügelgen and Wetter, 2000) nor $P2Y_{11}$.

Four findings argue against the 'classical' P_{2Y} purinoceptor: (i) ATP and MeSATP are about equipotent (Fig. 4B), in contrast to the potency order MeSATP >>ATP characteristic for this subtype (see Introduction); (ii) ADPβS, a potent agonist at P_{2Y} purinoceptors in other smooth muscle tissues (e.g. Bültmann et al., 1996) is almost inactive (Fig. 4B); (iii) on the other hand, UTP, which per definition (O'Connor et al., 1991) does not activate P_{2Y} purinoceptors (i.e. $P2Y_1$), causes prominent relaxation (Fig. 4A); (iv) suramin, PPADS and reactive red 2, which all block smooth muscle P_{2Y} purinoceptors (e.g. Bültmann and Starke, 1995; Windscheif et al., 1995; Bültmann et al., 1996; Johnson et al., 1996), failed to antagonize the response to ATP.

On the other hand, the site of action of the nucleotides is also distinct from the recombinant $P2Y_{11}$ receptor, which is not activated by UTP (Communi et al., 1997). Finally, the observed rank order of agonist potency (Fig. 4) is not compatible with any other recombinant P2 receptor presently known—P2Y as well as P2X. Specifically, $P2Y_1$ is ruled out be the relatively high potency of UTP and $P2Y_2$ by the relatively high potency of MeSATP (see Harden et al., 1998; Humphrey et al., 1998).

Apart from the unique rank order of agonist potency, two other findings indicate that the site mediating nucleotide-evoked relaxation of rat vas deferens may not be a P2

receptor at all: very high concentrations of the agonists were required (Fig. 4), and a broad spectrum of commonly used P2 receptor antagonists hardly attenuated the response to ATP. Only reactive blue 2, acid blue 25 and DIDS were effective antagonists (Fig. 5). Both the low potency of the agonists and the lack of effect of many antagonists might be explained by breakdown of the nucleotides by *ecto*-nucleotidases and an inhibition of this breakdown by the antagonists (cf. Crack et al., 1994; Bültmann et al., 1995, 1999b). However, among the antagonists that failed to attenuate the relaxation caused by ATP, only Evans blue and reactive red 2 were potent inhibitors of nucleotide breakdown in rat vas deferens (Bültmann et al., 1999b).

The present findings do not exclude a possible contribution of nucleotide P2 receptors to the relaxant effect of ATP. Nevertheless, an alternative explanation, namely, a site of action distinct from P2 receptors, has to be considered.

4.3. Ecto-protein kinases

Extracellular nucleotides serve as substrates for *ecto*-protein kinases (see Ehrlich et al., 1990; Redegeld et al., 1999). In the rat vas deferens, only nucleotides containing a hydrolysable and, hence, transferable terminal phosphate (or thiophosphate) group caused relaxation, while the stable analogues α,β-MeATP, β,γ-MeATP and ADPβS were almost inactive (Fig. 4). Moreover, the relaxation caused by ATP was markedly attenuated by three protein kinase inhibitors, H-7, staurosporine and K-252b (Fig. 6), which all block *ecto*-protein phosphorylation (Pirotton et al., 1992; Pawlowska et al., 1993; Muramoto et al., 1994; Fujii et al., 1995; Hogan et al., 1995). Therefore, the present findings may be evidence that the relaxation of rat vas deferens caused by ATP and related nucleotides is due to a phosphorylation reaction catalyzed by an *ecto*-protein kinase.

ATPγS is a substrate of protein kinases just like ATP (reviewed by Eckstein, 1985), but thiophosphorylation of proteins proceeds at a much slower rate than protein phosphorylation by ATP, and once incorporated thiophosphate is also removed more slowly. The observed time course (Fig. 1C), hence, is in accord with the suggested mode of action.

H-7, staurosporine and K-252b are nonselective inhibitors of several protein kinases including protein kinase A and protein kinase G (Rüegg and Burgess, 1989). In rat vas deferens, activation of protein kinase G does not lead to smooth muscle relaxation (Patel et al., 1997), so H-7, staurosporine and K-252b did not act against ATP by blocking protein kinase G. On the other hand, all three inhibitors markedly attenuated the relaxation of the rat vas deferens caused by adenosine (R. Bültmann; unpublished observation), presumably due to an inhibition of protein kinase A (cf. the effect of Rp-cAMPS in Fig. 2C). As adenosine contributes to the relaxant effect of ATP (see above), a small part of the effect of the three inhibitors against ATP (Fig. 6) may have been due to the inhibition of protein kinase A. However, the finding that H-7, staurosporine and K-252b were all considerably more effective than the selective protein kinase A inhibitor Rp-cAMPS (particularly against high concentrations of ATP; Fig. 6) indicates an additional site of inhibition, possibly an *ecto*-protein kinase.

In the present study, no attempt was made to measure the phosphorylation of membrane proteins directly (cf. Ehrlich et al., 1990). In the guinea-pig vas deferens, Lamport-Vrana et al. (1991) demonstrated phosphorylation or thiophosphorylation of a 19- to 21-kD membrane protein upon addition of [γ-^{32}P]ATP or [^{35}S]ATPγS, respectively, indicating an *ecto*-kinase activity. However, the incorporation of label was rapid and declined to near baseline values within 60 s, a time course markedly different from the nucleotide-evoked relaxation in rat vas deferens (Fig. 1).

4.4. Conclusion

In summary, the present results confirm the previously observed relaxation of the K^+-contracted vas deferens by ATP and other nucleotides. Pharmacological analysis indicates that adenosine, derived from the enzymatic breakdown of ATP, contributes to the relaxant effect of the nucleotide by activation of smooth muscle adenosine receptors. However, the main part of the response to ATP is mediated by a site distinct from the adenosine receptor. The pharmacological properties of this site differ from known P2 receptor subtypes. The relaxation caused by exogenous — and, possibly, also endogenous (Bültmann and Starke, 2001) — ATP may be due to a phosphoryl transfer catalyzed by an *ecto*-protein kinase, though direct evidence for this suggested mode of action is lacking.

Acknowledgements

We thank Bayer for suramin. This study was supported by the Deutsche Forschungsgemeinschaft (Sta 149/1-2).

References

Bailey, S.J., Hourani, S.M.O., 1994. Differential effects of suramin on P_2-purinoceptors mediating contraction of the guinea-pig vas deferens and urinary bladder. Br. J. Pharmacol. 112, 219–225.

Boland, B., Himpens, B., Vincent, M.F., Gillis, J.M., Casteels, R., 1992. ATP activates P_{2x}-contracting and P_{2y}-relaxing purinoceptors in the smooth muscle of mouse vas deferens. Br. J. Pharmacol. 107, 1152–1158.

Brownhill, V.R., Hourani, S.M.O., Kitchen, I., 1996. Differential distribution of adenosine A_2 receptors in the epididymal and prostatic portions of the rat vas deferens. Eur. J. Pharmacol. 303, 87–90.

Bruns, R.F., Lu, G.H., Pugsley, T.A., 1986. Characterization of the A_2 adenosine receptor labeled by [^{3}H]NECA in rat striatal membranes. Mol. Pharmacol. 29, 331–346.

Bültmann, R., Starke, K., 1995. Reactive red 2: a P_{2Y}-selective purinocep-

tor antagonist and an inhibitor of ecto-nucleotidase. Naunyn-Schmiedeberg's Arch. Pharmacol. 352, 477–482.

Bültmann, R., Starke, K., 2001. Nucleotide-evoked relaxation of rat vas deferens—a possible role for endogenous ATP released upon α_1-adrenoceptor stimulation. Eur. J. Pharmacol. 422, 197–202.

Bültmann, R., Driessen, B., Gonçalves, J., Starke, K., 1995. Functional consequences of inhibition of nucleotide breakdown in rat vas deferens: a study with Evans blue. Naunyn-Schmiedeberg's Arch. Pharmacol. 351, 555–560.

Bültmann, R., Dudeck, O., Starke, K., 1996. Evaluation of P_2-purinoceptor antagonists at two relaxation-mediating P_2-purinoceptors in guinea-pig taenia coli. Naunyn-Schmiedeberg's Arch. Pharmacol. 353, 445–451.

Bültmann, R., Klebroff, W., Starke, K., 1999a. A contraction-mediating receptor for UTP, presumably $P2Y_2$, in rat vas deferens. Naunyn-Schmiedeberg's Arch. Pharmacol. 360, 196–201.

Bültmann, R., Trendelenburg, M., Tuluc, F., Wittenburg, H., Starke, K., 1999b. Concomitant blockade of P2X-receptors and ecto-nucleotidases by P2-receptor antagonists: functional consequences in rat vas deferens. Naunyn-Schmiedeberg's Arch. Pharmacol. 359, 339–344.

Burnstock, G., Kennedy, C., 1985. Is there a basis for distinguishing two types of P_2-purinoceptor? Gen. Pharmacol. 16, 433–440.

Burnstock, G., Hopwood, A.M., Hoyle, C.H.V., Reilly, W.M., Saville, V.L., Stanley, M.D.A., Warland, J.J.I., 1986. Reactive blue-2 selectively antagonises the relaxant responses to ATP and its analogues which are mediated by the P_{2y} purinoceptor. Br. J. Pharmacol. 89, 857P.

Clanachan, A.S., Johns, A., Paton, D.M., 1977. Presynaptic inhibitory actions of adenine nucleotides and adenosine on neurotransmission in the rat vas deferens. Neuroscience 2, 597–602.

Communi, D., Govaerts, C., Parmentier, M., Boeynaems, J.M., 1997. Cloning of a human purinergic P2Y receptor coupled to phospholipase C and adenylyl cyclase. J. Biol. Chem. 272, 31969–31973.

Crack, B.E., Beukers, M.W., McKechnie, K.C.W., Ijzerman, A.P., Leff, P., 1994. Pharmacological analysis of ecto-ATPase inhibition: evidence for combined enzyme inhibition and receptor antagonism in P_{2X}-purinoceptor ligands. Br. J. Pharmacol. 113, 1432–1438.

Crema, A., Frigo, G.M., Lecchini, S., Manzo, L., Onori, L., Tonini, M., 1983. Purine receptors in the guinea-pig internal anal sphincter. Br. J. Pharmacol. 78, 599–603.

Eckstein, F., 1985. Nucleoside phosphorothioates. Annu. Rev. Biochem. 54, 367–402.

Ehrlich, Y.H., Hogan, M.V., Pawlowska, Z., Naik, U., Kornecki, E., 1990. Ectoprotein kinase in the regulation of cellular responsiveness to ATP. Ann. N. Y. Acad. Sci. 603, 401–416.

Fedan, J.S., Hogaboom, G.K., Westfall, D.P., O'Donnell, J.P., 1982. Comparison of contractions of the smooth muscle of the guinea-pig vas deferens induced by ATP and related nucleotides. Eur. J. Pharmacol. 81, 193–204.

Filtz, T.M., Li, Q., Boyer, J.L., Nicholas, R.A., Harden, T.K., 1994. Expression of a cloned P_{2Y}-purinergic receptor that couples to phospholipase C. Mol. Pharmacol. 46, 8–14.

Fujii, S., Kato, H., Furuse, H., Ito, K.I., Osada, H., Hamaguchi, T., Kuroda, Y., 1995. The mechanism of ATP-induced long-term potentiation involves extracellular phosphorylation of membrane proteins in guinea-pig hippocampal CA1 neurons. Neurosci. Lett. 187, 130–132.

Furchgott, R.F., 1972. The classification of adrenoceptors (adrenergic receptors): an evaluation from the standpoint of receptor theory. In: Blaschko, H., Muscholl, E. (Eds.), Catecholamines. Handbook of Experimental Pharmacology, vol. 33. Springer, Berlin, pp. 283–335.

Gailly, P., Boland, B., Paques, C., Himpens, B., Casteels, R., Gillis, J.M., 1993. Post-receptor pathway of the ATP-induced relaxation in smooth muscle of the mouse vas deferens. Br. J. Pharmacol. 110, 326–330.

Harden, T.K., Barnard, E.A., Boeynaems, H.M., Burnstock, G., Dubyak, G., Hourani, S.M.O., Insel, P.A., 1998. P2Y receptors. The IUPHAR Compendium of Receptor Characterization and Classification. IUPHAR media, London, pp. 209–217.

Hay, D.W.P., Wadsworth, R.M., 1982. Effects of some organic calcium antagonists and other procedures affecting Ca^{2+} translocation on KCl-induced contractions in the rat vas deferens. Br. J. Pharmacol. 76, 103–113.

Hogan, M.V., Pawlowska, Z., Yang, H.A., Kornecki, E., Ehrlich, Y.H., 1995. Surface phosphorylation by ecto-protein kinase C in brain neurons: a target for Alzheimer's β-amyloid peptides. J. Neurochem. 65, 2022–2030.

Hourani, S.M.O., Nicholls, J., Lee, B.S.S., Halfhide, E.J., Kitchen, I., 1993. Characterization and ontogeny of P_1-purinoceptors on rat vas deferens. Br. J. Pharmacol. 108, 754–758.

Huidobro-Toro, J.P., Parada, S., 1989. Pharmacological characterization of A1 and A2-adenosine receptors in the rat vas deferens neuroeffector junction. In: Ribeiro, J.A. (Ed.), Adenosine Receptors in the Nervous System. Taylor and Francis, London, p. 199.

Humphrey, P.P.A., Khakh, B.S., Kennedy, C., King, B.F., Burnstock, G., 1998. P2X receptors. The IUPHAR Compendium of Receptor Characterization and Classification. IUPHAR media, London, pp. 195–208.

Johnson, C.R., Charlton, S.J., Hourani, S.M.O., 1996. Responses of the longitudinal muscle and the muscularis mucosae of the rat duodenum to adenine and uracil nucleotides. Br. J. Pharmacol. 117, 823–830.

Kennedy, C., Leff, P., 1995. How should P_{2X} purinoceptors be classified pharmacologically. Trends Pharmacol. Sci. 16, 168–174.

Khakh, B.S., Surprenant, A., Humphrey, P.P.A., 1995. A study on P_{2X} purinoceptors mediating the electrophysiological and contractile effects of purine nucleotides in rat vas deferens. Br. J. Pharmacol. 115, 177–185.

Lamport-Vrana, S.J., Vrana, K.E., Fedan, J.S., 1991. Involvement of ecto-phosphoryl transfer in contractions of the smooth muscle of the guinea pig vas deferens to adenosine 5′ -triphosphate. J. Pharmacol. Exp. Ther. 258, 339–348.

Martin, P.L., May, J.M., 1994. Identification and functional characterization of A_1 and A_2 adenosine receptors in the rat vas deferens: a comparison with A_1 receptors in guinea pig left atrium and A_2 receptors in guinea pig aorta. J. Pharmacol. Exp. Ther. 269, 1228–1235.

Muramoto, K., Taniguchi, H., Kawahara, M., Kobayashi, K., Nonomura, Y., Kuroda, Y., 1994. A substrate of ecto-protein kinase is microtubule-associated protein 1B in cortical cell cultures undergoing synaptogenesis. Biochem. Biophys. Res. Commun. 205, 1467–1473.

O'Connor, S.E., Dainty, I.A., Leff, P., 1991. Further subclassification of ATP receptors based on agonist studies. Trends Pharmacol. Sci. 12, 137–141.

Patel, A.I., Hennan, J.K., Diamond, J., 1997. Activation of guanosine 3′ , 5′ -cyclic monophosphate (cGMP)-dependent protein kinase in rat vas deferens and distal colon is not accompanied by inhibition of contraction. J. Pharmacol. Exp. Ther. 283, 894–900.

Pawlowska, Z., Hogan, M.V., Kornecki, E., Ehrlich, Y.H., 1993. Ecto-protein kinase and surface protein phosphorylation in PC12 cells: interactions with nerve growth factor. J. Neurochem. 60, 678–686.

Pirotton, S., Boutherin-Falson, O., Robaye, B., Boeynaems, J.M., 1992. Ecto-phosphorylation on aortic endothelial cells. Biochem. J. 285, 585–591.

Redegeld, F.A., Caldwell, C.C., Sitkovsky, M.V., 1999. Ecto-protein kinases: ecto-domain phosphorylation as a novel target for pharmacological manipulation? Trends Pharmacol. Sci. 20, 453–459.

Rüegg, U.T., Burgess, G.M., 1989. Staurosporine, K252 and UCN-01: potent but nonspecific inhibitors of protein kinases. Trends Pharmacol. Sci. 10, 218–220.

Tennant, J.P., Pearson, A., Hournai, S.M.O., 1999. Effects of noradrenaline, the calcium ionophore A23187, forskolin, sodium nitroprusside and glibenclamide on the degradation of extracellular adenosine 5′ -triphosphate by the rat isolated vas deferens. J. Auton. Pharmacol. 19, 167–171.

Tokuyama, Y., Hara, M., Jones, E.M.C., Fan, Z., Bell, G.I., 1995. Cloning of rat and mouse P_{2Y} purinoceptors. Biochem. Biophys. Res. Commun. 211, 211–218.

Triggle, C.R., Swamy, V.C., Triggle, D.J., 1979. Calcium antagonists and contractile responses in rat vas deferens and guinea pig ileal smooth muscle. Can. J. Physiol. Pharmacol. 57, 804–818.

Tuluc, F., Bültmann, R., Glänzel, M., Frahm, A.W., Starke, K., 1998. P2-receptor antagonists: IV. Blockade of P2-receptor subtypes and ecto-nucleotidases by compounds related to reactive blue 2. Naunyn-Schmiedeberg's Arch. Pharmacol. 357, 111–120.

Von Kügelgen, I., Wetter, A., 2000. Molecular pharmacology of P2Y-receptors. Naunyn-Schmiedeberg's Arch. Pharmacol. 362, 310–323.

Von Kügelgen, I., Bültmann, R., Starke, K., 1990. Interaction of adenine nucleotides, UTP and suramin in mouse vas deferens: suramin-sensitive and suramin-insensitive components in the contractile effect of ATP. Naunyn-Schmiedeberg's Arch. Pharmacol. 342, 198–205.

Waud, D.R., 1976. Analysis of dose-response relationships. In: Narahashi, T., Waud, D.R., Bianchi, C.P. (Eds.), Advances in General and Cellular Pharmacology, vol. 1. Plenum, New York, pp. 145–178.

Webb, T.E., Simon, J., Krishek, B.J., Bateson, A.N., Smart, T.G., King, B.F., Burnstock, G., Barnard, E.A., 1993. Cloning and functional expression of a brain G-protein-coupled ATP receptor. FEBS 324, 219–225.

Windscheif, U., Pfaff, O., Ziganshin, A.U., Hoyle, C.H.V., Bäumert, H.G., Mutschler, E., Burnstock, G., Lambrecht, G., 1995. Inhibitory action of PPADS on relaxant responses to adenine nucleotides or electrical field stimulation in guinea-pig taenia coli and rat duodenum. Br. J. Pharmacol. 115, 1509–1517.