ATP modulates noradrenaline release by activation of inhibitory P2Y-receptors and facilitatory P2X-receptors in the rat vas deferens

Glória Queiroz, Carlos Talaia and Jorge Gonçalves

Laboratório de Farmacologia, Faculdade de Farmácia, CEQOFFUP
Universidade do Porto, Porto, Portugal
Modulation of noradrenaline release by P2Y and P2X receptors

Corresponding author: Jorge Gonçalves, Laboratório de Farmacologia, Faculdade de Farmácia, Universidade do Porto, Rua Aníbal Cunha, 164, 4050-047 Porto, Portugal
Tel: + 351 222 2078 932 Fax: + 351 222 078 969 E-mail: jorge.goncalves@ff.up.pt

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ABBREVIATIONS: ARL 67156, 6-N,N-diethyl-D-β,γ-dibromomethylene 5′-triphosphate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; β,γ-imidoATP, β,γ-imidoadenosine 5′-triphosphate; 2-MeSAMP, 2-methylthioadenosine 5′-monophosphate; 2-MeSADP, 2-methylthioadenosine-5′-diphosphate; 2-MeSATP, 2-methylthioadenosine-5′-triphosphate; α,β-meATP, α,β-methyleneadenosine 5′-triphosphate; β,γ-meATP, β,γ-methyleneadenosine 5′-triphosphate; MRS 2179, 2′-deoxy-N6-methyladenosine 3′,5′-bisphosphate; NF 279, 8,8′-[carbonylbis(iminono-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)]bis-1,3,5-naphthalenetrisulfonic acid; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; PPNDS, pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulphonate); RB2, reactive blue 2; ZM 241385, 4-(2[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)-phenol.
ABSTRACT

The role of ATP on the modulation of noradrenaline release elicited by electrical stimulation (100 pulses/8 Hz) was studied in the prostatic portion of rat vas deferens pre-incubated with [³H]-noradrenaline.

In the presence of P1-antagonists, the nucleotides 2-methylthioadenosine-5'-triphosphate (2-MeSATP), 2-methylthioadenosine 5'-diphosphate (2-MeSADP), ADP and ATP decreased electrically evoked tritium overflow up to 44 %, with the following order of potency: 2-MeSATP > 2-MeSADP > ADP ≥ ATP. The P2Y-antagonists RB2 (reactive blue 2) and 2-MeSAMP (2-methylthioadenosine 5'-monophosphate) increased whereas the P2X-antagonist PPNDS (pyridoxal-5'-phosphate-6-(2'-naphthylazo-6'-nitro-4',8'-disulphonate), decreased evoked tritium overflow. The inhibitory effect of 2-MeSATP was antagonized by RB2 (10 µM) and by 2-MeSAMP (10 µM) but not by the selective P2Y1-receptor antagonist MRS 2179 (2'-deoxy-N⁶-methyladenosine 3',5'-bisphosphate, 10 µM).

When, besides P1-receptors, inhibitory P2Y-receptors where blocked with RB2, α,β-meATP (α,β-methyleneadenosine 5'-triphosphate), β,γ-imidoATP (β,γ-imidoadenosine 5'-triphosphate), β,γ-meATP (β,γ-methyleneadenosine 5'-triphosphate), 2-MeSATP and ATP enhanced tritium overflow up to 140 %, with the following order of potency: α,β-meATP > 2-MeSATP = ATP = β,γ-meATP ≥ β,γ-imidoATP. The facilitatory effects of α,β-MeATP and β,γ-imidoATP were prevented PPNDS. Under the same conditions, apyrase attenuated whereas the ectonucleotidase inhibitor 6-N,N-diethyl-D-β,γ-dibromomethylene 5'-triphosphate enhanced tritium overflow, an effect that was prevented by PPNDS.

In the prostatic portion of the rat vas deferens, endogenous ATP exerts a dual and opposite modulation of noradrenaline release: an inhibition through activation of P2Y-receptors with a pharmacological profile similar to that of the P2Y12- and P2Y13-receptors and a facilitation through activation of P2X-receptors with a pharmacological profile similar to that of P2X1- and P2X3- or PX2/P2X3-receptors.
In the sympathetic nervous system ATP is stored and released with noradrenaline from postganglionic nerve terminals and acts not only as a transmitter (Burnstock, 1990; Westfall et al., 2002) but also as a presynaptic modulator (von Kügelgen et al., 1999). The released ATP exerts effects on nerve terminals and on postsynaptic cells, by activation of membrane receptors named P2-receptors. P2-receptors are a family of receptors that comprises two groups: ionotropic P2X-receptors and metabotropic P2Y-receptors (Ralevic and Burnstock, 1998). Seven subtypes of P2X subunits, named P2X1-P2X7, have been identified at the molecular level, which by homo- or hetero-oligomeric assembly may form functional receptor-channels (Torres et al., 1999). The P2Y-receptor group currently includes the cloned mammalian receptors P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and the UDP-glucose receptor now re-named P2Y14 (Ralevic and Burnstock, 1998; Abbracchio et al., 2003). The P2Y-receptors can be grouped according to their sensitivity to purines and/or to pyrimidines into three functional groups: (P2Y1, P2Y12 and P2Y13), ii) selective pyrimidinoceptors (P2Y6), iii) receptors with mixed selectivity (P2Y2, P2Y4 and P2Y11; see White et al., 2003).

In many sympathetic innervated tissues, adenine nucleotides such as ATP and its analogues have been shown to inhibit action-potential evoked release of noradrenaline by direct activation of presynaptic inhibitory P2Y-receptors (von Kügelgen et al., 1999). In addition to presynaptic inhibitory P2Y-receptors, sympathetic nerve terminals may also be endowed with excitatory P2-receptors. Cultured rat sympathetic neurons (Boehm, 1999; Nörenberg et al., 1999) and postganglionic sympathetic nerves that innervate the right atrium and ileum (Sperlágh et al., 1991; 2000) express P2X-receptors that are present on axon terminals and mediate an enhancement of noradrenaline release.

In rat vas deferens, a sympathetic innervated tissue, a P2-receptor-mediated inhibition of noradrenaline release has already been demonstrated (Kurz et al., 1993). However, the receptor subtype involved has not been identified. Furthermore,
immunohistochemical studies have shown that, in this tissue, P2X-receptors are also present on nerve terminals (Vulchanova et al., 1996; Lee et al. 2000) but their putative involvement on modulation of noradrenaline release was never investigated.

Therefore, the aims of the present study were: i) to study the role of ATP on modulation of noradrenaline release, ii) to characterize pharmacologically the P2-receptor subtypes that mediate the inhibitory effects of ATP on noradrenaline release and iii) to investigate whether P2X-receptors also participate on the modulation of noradrenaline release in the rat vas deferens. In this study, only the prostatic portion of rat vas deferens was used because in this preparation the importance of ATP as transmitter is particularly relevant (Sneddon and Machaly, 1992).
Experimental Protocol. Adult male Wistar rats (290 - 340 g; IBMC, Porto, Portugal) were used. Handling and care of animals were conducted according to the EU guiding principles in animal research (86/609/EU). Animals were killed by cervical dislocation and exsanguination. Prostatic halves of vas deferens were dissected out, cleaned of connective tissue and divided longitudinally into preparations. Eight tissue preparations were then incubated in 2-ml medium containing 0.1 $\mu$M $[^{3}\text{H}]$-noradrenaline, for 40 min at 37ºC. Individual preparations were placed in superfusion chambers between platinum electrodes and superfused with $[^{3}\text{H}]$-noradrenaline-free medium at a rate of 1 ml min$^{-1}$. Successive 5-min samples of the superfusate were collected from $t = 55$ min onwards ($t = 0$ min being the start of superfusion). At the end of the experiments, tritium was determined in superfusate samples and in tissues by scintillation spectrometry (Beckman LS 6500, Beckman Instruments, Fullerton, USA). The medium contained (mM): NaCl 118.6, KCl 4.70, CaCl$_2$ 2.52, MgSO$_4$ 1.23, NaHCO$_3$ 25.0, glucose 10.0, ascorbic acid 0.3, disodium EDTA 0.031; was saturated with 95% O$_2$ - 5% CO$_2$ and kept at 37ºC. The superfusion medium also contained desipramine (400 nM; to inhibit neuronal uptake of noradrenaline) and yohimbine (1 $\mu$M; to block $\alpha_2$-autoreceptors).

Up to five identical periods of electrical stimulation were applied (Stimulator II, Hugo Sachs Elektronik, March-Hugstetten, Germany; constant current mode; rectangular pulses; 1-ms width; current strength 50 mA; voltage drop between electrodes 18 V cm$^{-1}$). The first, starting at $t = 30$ min ($S_0$) was not used for determination of tritium outflow. The subsequent periods ($S_1$ up to $S_4$), also consisting of 100 pulses at 8 Hz, started at $t = 60$ min with 30-min intervals. Concentration-response curves for P2-receptor agonists were obtained by adding the agonists at increasing concentration 8 min before $S_2$, $S_3$ and $S_4$ up to the end of each stimulation period. Concentration-response curves for P2-receptor...
antagonists were obtained by adding antagonists 20 min before S₂, S₃ and S₄ at increasing concentrations. When indicated, DPCPX (100 nM; to block adenosine A₁ receptors), ZM 241385 (100 nM; to block adenosine A₂ receptors) and RB2 (10 µM; to block P2Y-receptors) were added throughout superfusion.

Data Evaluation. The outflow of tritium was expressed as fraction of the tissue tritium content at the onset of the respective collection period (fractional rate of outflow, min⁻¹). Effects of drugs on basal tritium outflow were estimated by the bₙ/b₁ ratios and were expressed as percentage of the mean ratio obtained in the appropriate control; bₙ was the fractional rate of outflow in the 5-min period before S₂, S₃ and S₄ (b₂, b₃ and b₄, respectively) and b₁ was the fractional rate of outflow in the 5-min period before S₁. The overflow of tritium evoked by electrical stimulation was calculated as the difference between “total tritium outflow during the 10 min period after start of stimulation” and the estimated “basal outflow”, being expressed as percentage of tritium content of the tissue at the onset of stimulation. Effects of drugs added after S₁ on tritium overflow were evaluated as ratios of the overflow elicited by S₂, S₃ and S₄ (Sₙ) and the overflow elicited by S₁ (Sₙ/S₁). Sₙ/S₁ values obtained in individual experiments in which a test compound A was added after S₁ were calculated as percentage of the respective mean ratio in the appropriate control group (solvent instead of A). When interaction of drug A, added after S₁, and a drug B added either after S₁ or at the beginning of superfusion, was studied, the “appropriate control” was a group in which B alone was used.

Drugs. Levo-[ring-2,5,6-³H]-noradrenaline, specific activity 46.8 Ci mmol⁻¹ was from DuPont NEN (Garal, Lisboa, Portugal); adenosine-5'-diphosphate tetrasodium (ADP), adenosine-5'-triphosphate disodium (ATP), apyrase Grade VI (EC 3.6.1.5), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), desipramine hydrochloride, 2-methylthioadenosine-5'-diphosphate trisodium (2-MeSADP), 2-methylthioadenosine 5'-monophosphate triethylammonium (2-MeSAMP), 2-methylthioadenosine-5'-triphosphate tetrasodium (2-
MeSATP), β,γ-imidoadenosine 5’-triphosphate tetralithium (β,γ-imidoATP), α,β-methyleneadenosine 5’-triphosphate lithium (α,β-meATP), β,γ-methyleneadenosine 5’-triphosphate disodium (β,γ-meATP), suramin hexasodium, reactive blue 2 (basilen blue E-3G; RB2) and yohimbine hydrochloride were from Sigma (Sintra, Portugal); 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385), 8,8´-[carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)]bis-1,3,5-naphthalene-trisulfonic acid hexasodium (NF 279), 2´-deoxy-N6-methyladenosine 3´,5´-bisphosphate tetrammonium (MRS 2179), 6-N,N-diethyl-D-β,γ-dibromomethylene 5’-triphosphate triammonium (ARL 67156), pyridoxalphosphate-6-azophenyl-2´,4´-disulphonic acid tetrasodium (PPADS) and pyridoxal-5´-phosphate-6-(2´-naphthylazo-6´-nitro-4´,8´-disulphonate) tetrasodium (PPNDS) were from Tocris (Bristol, UK). Stock solutions up to 10 mM were made in DMSO or water and kept at –20 ºC. Solutions of drugs were prepared immediately before use and solvent was added to the superfusion medium in parallel control experiments.

Statistical Analysis. Results are presented as means ± S.E.M.; n is the number of tissue preparations. Effect of drugs on basal tritium outflow and evoked tritium overflow was tested for significance by an analysis of variance (ANOVA) followed by the Dunnett’s multiple comparison test. P values lower than 0.05 were taken to indicate significant differences.
RESULTS

**General Observations.** The fractional rate of basal tritium outflow and electrically evoked tritium overflow from prostatic portions of rat vas deferens in different experimental conditions are shown in Table 1. Blockade of adenosine receptors with the adenosine A1 receptor antagonist, DPCPX (Lohse et al., 1987) and with the adenosine A2A receptor antagonist, ZM 241385 (Poucher et al., 1995) did not change either basal tritium outflow or evoked tritium overflow. When, in addition to P1-antagonists, the P2Y-antagonist RB2 (10 μM) was added throughout superfusion, basal tritium outflow and evoked tritium overflow were increased (Table 1). Basal tritium outflow and evoked tritium overflow remained constant throughout the experiment regardless of the drugs added throughout superfusion, with b/n/b1 and S/n/S1 values close to unity (not shown). Basal tritium outflow was not changed by drugs added after S1, except by the P2-antagonists PPADS, PPNDS and RB2 (see below).

**Influence of P2-receptor agonists on tritium overflow.** Electrical stimulation of prostatic portion of rat vas deferens by 100 pulses/8Hz caused an overflow of tritium that was modified by several purine nucleotides. The nucleotides were tested in the presence of the P1-antagonists DPCPX (100 nM; to block adenosine A1 receptors) and ZM 241385 (100 nM; to block adenosine A2 receptors), to avoid contribution of these receptors to the effects of nucleotides. Under these conditions, α,β-meATP and β,γ-meATP slightly increased, β,γ-imidoATP did not change, whereas 2-MeSATP, 2-MeSADP, ADP and ATP decreased evoked tritium overflow in a concentration-dependent manner (Fig. 1). Adenosine (1 mM) only caused a small decrease of evoked tritium overflow (S2/S1 = 87 ± 3 %; n = 4; P < 0.05). The apparent order of potency of nucleotides that decreased evoked tritium overflow was: 2-MeSATP > 2-MeSADP > ADP ≥ ATP. As shown in Figure 2, the effect of 2-MeSATP was prevented by the non-selective P2Y-antagonist RB2 (10 μM) and
by the P2Y12- and P2Y13- antagonist 2-MeSAMP (10 µM; Hollopeter et al., 2001; Zhang et al., 2002) but not by the selective P2Y1-antagonist MRS 2179 (10 µM; Boyer et al., 1998).

**Influence of P2-receptor agonists on tritium overflow, in the presence of RB2.**

The results obtained with nucleotides suggest that, in addition to inhibitory P2Y-receptors, facilitatory P2-receptors may also be involved on the modulation of tritium overflow. Because there are no selective agonists for each of the P2-receptors, the effects of nucleotides were re-evaluated in the presence of 10 µM of RB2 (added at beginning of superfusion and kept throughout) to block inhibitory P2Y-receptors.

When inhibitory P2Y-receptors were blocked, α,β-meATP, β,γ-meATP, 2-MeSATP, β,γ-imidoATP and ATP, but not 2-MeSADP, caused a concentration-dependent increase on evoked tritium overflow (Fig. 3). The apparent order of potency of nucleotides that caused a facilitation of evoked tritium overflow was: α,β-meATP > 2-MeSATP = ATP = β,γ-meATP ≥ β,γ-imidoATP.

The facilitatory effects of α,β-meATP and β,γ-imidoATP were prevented by the P2X-antagonist PPNDS (3 µM; Lambrecht, 2000; Fig. 4) suggesting an involvement of facilitatory P2X-receptors on the modulation of evoked tritium overflow.

**Influence of P2-receptor antagonists on tritium overflow.** The effect of several P2-antagonists on evoked tritium overflow was also tested in order to investigate whether the ATP released was tonically activating the P2-receptors under the stimulation conditions used.

The P2-antagonists suramin and RB2 (3 – 30 µM) caused a concentration dependent increase on evoked tritium overflow whereas PPADS, PPNDS and RB2 (100 and 300 µM) caused a concentration-dependent decrease on the evoked tritium overflow (Table 2). They also decreased basal tritium outflow: PPADS (3 – 30 µM) up to 22 ± 2 % (n = 9; P < 0.01), PPNDS (1 – 10 µM) up to 37 ± 5 % (n = 6; P < 0.01) and RB2 (30 – 100 µM) up to
51 ± 3 % (n = 6; P < 0.01). The effects of RB2 and PPNDS on evoked tritium overflow were not changed when P1-receptors were blocked (100 nM DPCPX plus 100 nM ZM 241385) excluding a major contribution of endogenous adenosine to the effects of P2-antagonists (Fig. 5). Furthermore, the selective P2X1-antagonist NF 279 (Damer et al., 1998) decreased evoked tritium overflow, although much less than PPNDS, the P2Y12- and PY13-antagonist 2-MeSAMP increased whereas the selective P2Y1-antagonist MRS 2179 had no effect on tritium overflow (Fig. 5).

**Influence of apyrase and ARL 67156 on tritium overflow.** In order to confirm that the decrease on evoked tritium overflow caused by the more selective P2X-antagonists PPNDS and PPADS (see Lambrecht 2000) was due to blockade of a facilitation of noradrenaline release mediated by endogenous ATP, the effect of apyrase (an enzyme that metabolises ATP; Zimmermann et al., 1999) and of the ectonucleotidase inhibitor 6-N,N-diethyl-D-β,γ-dibromomethylene 5'-triphosphate (ARL 67156; Crack et al., 1995) were investigated. Apyrase and ARL 67156 were tested in the presence of DPCPX (100 nM), ZM 241385 (100 nM) and RB2 (10 µM; the concentration that prevented the inhibitory effect of 2-MeSATP and caused a concentration-dependent increase on tritium overflow, see Figs. 2 and 5), in order to remove the influence of P1-receptors and inhibitory P2Y-receptors, respectively. Apyrase (5 U/ml) decreased whereas ARL 67156 (50 µM) increased evoked tritium overflow (Fig. 6). The facilitatory effect of ARL 67156 was prevented by PPNDS (3 µM; Fig. 6) supporting the observation that endogenous ATP may increase evoked tritium overflow by activation of facilitatory P2X-receptors.
The electrically evoked tritium overflow from tissue preparations of rat vas deferens pre-incubated with [3H]-noradrenaline was assumed to reflect action potential-evoked neuronal release of noradrenaline. In accordance with previous findings (Kurz et al., 1993), noradrenaline release was inhibited by ATP and by other nucleotides such as 2-MeSATP, and enhanced by the P2-antagonists suramin and RB2. It is unlikely that effects of ATP and other nucleotides tested on noradrenaline release are due to adenosine because they were still observed in the presence of the P1-antagonists DPCPX and ZM 241385 (see Fig. 1). Therefore, changes in tritium overflow caused by nucleotides and P2-antagonists are, most likely, mediated by prejunctional P2-receptors, with the inhibitory effects being mediated by prejunctional P2Y-receptors.

**Inhibitory P2Y-receptors.** From the P2Y-receptors already cloned, only the P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14 subtypes have been shown to occur in mammalian tissues (see Ralevic and Burnstock, 1998; Abbracchio et al., 2003). Involvement of P2Y2, P2Y4, P2Y6, P2Y11 subtypes on the inhibition of noradrenaline release may be excluded by the following reasons: i) P2Y2 is only weakly activated by 2-MeSATP, 2-MeSADP and ADP (Nicholas et al., 1996), nucleotides that caused a marked inhibition of noradrenaline release; ii) P2Y4 is insensitive to suramin (Charlton et al., 1996), an antagonist that caused a facilitation of noradrenaline release in the present study, possibly by preventing a tonic activation of inhibitory P2Y-receptors; iii) P2Y6 is selective for uridine nucleotides, is very weakly activated by 2-MeSATP and is not activated by ATP and ADP (Nicholas et al., 1996) whereas, in the present study, 2-MeSATP was the most potent compound at the inhibitory P2-receptors; iv) the P2Y11 is sensitive to suramin and RB2 but is not activated by ADP (Communi et al., 1997;1999).
that, in the present study, caused a concentration-dependent inhibition of noradrenaline release.

The P2Y1, P2Y12 and P2Y13 subtypes are receptors with a very similar agonist profile. The order of potency of nucleotides that activate these receptors is: 2-MeSADP ≥ 2-MeSATP > ADP ≥ ATP (Boyer et al., 1996; Hollopeter et al., 2001; Zhang et al., 2002). This agonist profile is very similar to that observed for inhibitory P2Y-receptors in the present study: 2-MeSATP > 2-MeSADP > ADP ≥ ATP. Therefore, P2Y1, P2Y12 and P2Y13 subtypes are the most serious candidates to mediate that inhibition of noradrenaline release.

P2Y1-receptors are blocked by MRS 2179, which is selective for this receptor subtype (Boyer et al., 1998) and also by RB2, suramin and PPADS, with similar potencies (Boyer et al., 1994). Furthermore, it has been shown that P2Y1-receptors can close N-type Ca^{2+}-channels in neurons (Filippov et al., 2000), a mechanism that may explain the inhibitory effect of nucleotides on transmitter release. The lack of antagonism by MRS 2179 of inhibitory effects caused by 2-MeSATP, combined with the observation that, per se, MRS 2179 did not change noradrenaline release whereas PPADS decreased noradrenaline release, exclude the involvement of P2Y1-receptors on inhibition of noradrenaline release in the prostatic portion of the rat vas deferens. Furthermore, the observation that nucleotides, such as 2-MeSATP and 2-MeSADP, decreased noradrenaline release even in the presence of the adenosine A_1-receptor antagonist DPCPX (at a concentration that is about 200 times higher than its affinity constant at the adenosine A_1 receptor; Lohse et al., 1987) excludes the involvement of the recently described heteromers of P2Y1- and adenosine A_1 receptors (Yoshioka et al., 2001) or the P3-receptor (Forsyth et al., 1991) on the inhibition of noradrenaline release caused by these nucleotides.
P2Y12 and P2Y13 subtypes are both blocked by 2-MeSAMP, with similar potency (Hollopeter at 2001; Zhang et al., 2002). Since 2-MeSAMP prevented the inhibitory effects of 2-MeSATP, the PY12- and/or the P2Y13-receptor-subtypes are the strongest candidates to mediate an inhibition of noradrenaline release in the prostatic portion of rat vas deferens. Contribution of P2Y12-receptors can be questioned because ATP is an antagonist at P2Y12-receptors (Hollopeter at 2001). However, the involvement of the P2Y12-receptors cannot be completely excluded because there is the possibility that ATP may be partly metabolized to ADP that is an agonist at these receptors.

Participation of P2Y13-receptors, and eventually P2Y12-receptors, on modulation of noradrenaline release is compatible with their known distribution and coupling system. P2Y13-receptors are widely expressed (Zhang et al., 2002) and like P2Y12-receptors, are negatively coupled to adenylate cyclase through activation of a G<sub>i</sub> protein (Hollopeter et al., 2001; Zhang et al., 2002). Recently, several studies have shown that P2Y12 and PY13-like receptors may mediate an inhibition of neuronal Ca<sup>2+</sup>-channels (Powell et al., 2000; Unterberg et al., 2002; Kublick and von Kügelgen, 2002; Kubista et al., 2003). This signalling pathway is also activated by other presynaptic receptors that inhibit transmitter release (see Boehm and Kubista, 2002).

**Facilitatory P2X-receptors.** Our results also show that some purine nucleotides including ATP may also enhance noradrenaline release in the prostatic portion of rat vas deferens. This conclusion is supported by the observation that nucleotides, such as α,β-meATP, increased noradrenaline release, even when inhibitory P2Y-receptors were blocked, and by the observation that the PX-antagonists PPADS, PPNDS and NF 279 decreased noradrenaline release, an effect that is most likely due to an attenuation of a release enhancing effect of endogenous ATP. Evidence that endogenous ATP was activating facilitatory P2-receptors was obtained by changing the levels of endogenous ATP under conditions of low influence of inhibitory P2Y-receptors. The ATP metabolizing
enzyme apyrase decreased noradrenaline release, most likely by preventing the release enhancing effects of endogenous ATP whereas the ecto-ATPase inhibitor ARL 67156 which prevents ATP degradation, increased noradrenaline release, most likely by favoring the release enhancing effects of endogenous ATP.

P2Y11-receptors can couple to intracellular pathways that may lead to a facilitation of transmitter release (Communi et al., 1997) and can be activated by $\alpha,\beta$-meATP (van der Weyden et al., 2000) but their involvement on the facilitation of noradrenaline release it is unlikely because they are blocked RB2 but not by PPADS (Communi et al., 1999; van der Weyden et al., 2000). Therefore, the P2-receptors that mediate a facilitation of noradrenaline release seem to belong to the P2X-receptor-subtype, as observed in other sympathetic sympathetic innervated tissues (Sperlăgh et al., 2000) and cultured sympathetic neurons (Boehm, 1999).

From all functional P2X-receptors known, homomeric P2X1- or P2X3-receptors and heteromeric receptors composed of P2X2/P2X3, P2X1/P2X5 and P2X4/P2X6 subunits are activated by $\alpha,\beta$-meATP (Nörenberg and Illes, 2000). Immunohistochemical studies have shown that only the P2X1, P2X2 and P2X3 subunits are expressed in rat vas deferens (Lee et al., 2000) and some of them, namely the P2X2 and P2X3 subunits are found on nerves fibers and nerve terminals (Vulchanova et al., 1996; Lee et al., 2000).

The antagonist profile of facilitatory P2-receptors that modulate noradrenaline release is similar to that described for homomeric P2X1 or P2X3 receptors and for heteromeric P2X2/P2X3 receptors (see Lambrecht, 2000). The agonist profile observed: $\alpha,\beta$-meATP $> 2$-MeSATP $= ATP = \beta,\gamma$-meATP $\geq \beta,\gamma$-imidoATP, is slightly different from that described for homomeric P2X1 or P2X3 receptors and heteromeric P2X2/P2X3 receptors (2-MeSATP $\geq$ ATP $> \alpha,\beta$-meATP; Nörenberg and Illes, 2000). This discrepancy may not indicate the presence of a different P2X-receptor subtype but be just a consequence of the coexistence of P2Y- and P2X-receptors that are activated by the
same nucleotides; 2-MeSATP may also activate inhibitory receptors disturbing a clear
definition of the order of potency of agonists at facilitatory P2X-receptors.

In conclusion, endogenous ATP exerts a dual and opposite modulation of
noradrenaline release in the prostatic portion of the rat vas deferens: an inhibition through
activation of P2Y-receptors with a pharmacological profile similar to that of the recently
cloned P2Y12- and/or P2Y13-subtypes and a facilitation through activation of P2X-
receptors that have a pharmacological profile similar to that of homomeric P2X1 and P2X3
or heteromeric P2X2/P2X3-receptors.
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Figure 1. Effects of P2-receptor agonists on evoked tritium overflow from prostatic portion of rat vas deferens in the presence of the P1-antagonists DPCPX and ZM 241385. Tissue preparations were incubated with $[^3]H$-noradrenaline for 40 min and electrically stimulated with 5 trains of 100 pulses/8Hz ($S_0 – S_4$). DPCPX (100 nM) and ZM 241385 (100 nM) were added at the beginning of superfusion and kept throughout. P2-receptor agonists were added 8 min before $S_2$, $S_3$ and $S_4$ at increasing concentrations and kept until the end of the respective stimulation period. For evaluation of effects of drugs, $S_n/S_1$ ratios obtained in the presence of agonists were expressed as percentage of the average of the corresponding $S_n/S_1$ control value (see Methods). Values are means ± S.E.M. from 4 – 7 tissue preparations. Significant differences from respective control (solvent): * $P < 0.05$ and ** $P < 0.01$.

Figure 2. Effect of 2-MeSATP on evoked tritium overflow from prostatic portion of rat vas deferens in the absence and in the presence of the P2Y-antagonists RB2, 2-MeSAMP and MRS 2179. Tissue preparations were incubated with $[^3]H$-noradrenaline for 40 min and electrically stimulated with 5 trains of 100 pulses/8Hz ($S_0 – S_4$). The P1-antagonists DPCPX (100 nM) and ZM 241385 (100 nM) were added at the beginning of superfusion and kept throughout. RB2 (10 $\mu$M), 2-MeSAMP (10 $\mu$M) and MRS 2179 (10 $\mu$M) were added 20 before $S_2$ and kept throughout. 2-MeSATP was added 8 min before $S_2$, $S_3$ and $S_4$ at increasing concentrations and kept until the end of the respective stimulation period. For evaluation of effects of drugs, $S_n/S_1$ ratios obtained in the presence of 2-MeSATP were expressed as percentage of the average of the corresponding $S_n/S_1$ control value (see Methods). Values are means ± S.E.M. from 4 – 10 tissue preparations. Significant
differences from respective control (solvent): * $P < 0.05$ and ** $P < 0.01$; from the effect of 2-MeSATP alone, † $P < 0.05$ and ‡ $P < 0.01$.

Figure 3. Effects of P2-receptor agonists on tritium overflow from prostatic portion of rat vas deferens in the presence of the P1-antagonists DPCPX and ZM 241385 and of the P2Y-antagonist RB2. Tissue preparations were incubated with $[3^H]$-noradrenaline for 40 min and electrically stimulated with 5 trains of 100 pulses/8Hz (S0 – S4). DPCPX (100 nM), ZM 241385 (100 nM) and RB2 (10 µM) were added at the beginning of superfusion and kept throughout. P2-receptor agonists were added 8 min before S2, S3 and S4 at increasing concentrations and kept until the end of the respective stimulation period. For evaluation of effects of drugs, $S_n/S_1$ ratios obtained in the presence of agonists were expressed as percentage of the average of the corresponding $S_n/S_1$ control value (see Methods). Values are means ± S.E.M. from 4 – 10 tissue preparations. Significant differences from respective control (solvent): * $P < 0.05$ and ** $P < 0.01$.

Figure 4. Effects of $\alpha,\beta$-meATP and $\beta,\gamma$-imidoATP on evoked tritium overflow from prostatic portion of rat vas deferens in the absence (closed symbols) and in the presence of the P2X-antagonist PPNDS (3 µM; open symbols). Tissue preparations were incubated with $[3^H]$-noradrenaline for 40 min and electrically stimulated with 5 trains of 100 pulses/8Hz (S0 – S4). The P1-antagonists DPCPX (100 nM) and ZM 241385 (100 nM) and the P2Y-antagonist RB2 (10 µM) were added at the beginning of superfusion and kept throughout. PPNDS (3 µM) was added 20 min before S2 and kept throughout. $\alpha,\beta$-meATP and $\beta,\gamma$-imidoATP were added 8 min before S2, S3 and S4 at increasing concentrations and kept until the end of the respective stimulation period. For evaluation of effects of drugs, $S_n/S_1$ ratios obtained in the presence of $\alpha,\beta$-meATP and $\beta,\gamma$-imidoATP were expressed as percentage of the average of the corresponding $S_n/S_1$ control value (see Methods). Values
are means ± S.E.M. from 5 – 10 tissue preparations. Significant differences from respective control (solvent): * \( P < 0.05 \) and ** \( P < 0.01 \); from the effect of \( \alpha,\beta\)-meATP and \( \beta,\gamma\)-imidoATP alone, ++ \( P < 0.01 \).

Figure 5. Effects of P2-antagonists on evoked tritium overflow from prostatic portion of rat vas deferens in the presence of the P1-antagonists DPCPX and ZM 241385. Tissue preparations were incubated with \[^{3}H\]noradrenaline for 40 min and electrically stimulated with 5 trains of 100 pulses/8Hz (S0 – S4). DPCPX (100 nM) and ZM 241385 (100 nM) were added at the beginning of superfusion and kept throughout. P2-antagonists were added 20 min before S2, S3 and S4 at increasing concentrations. For evaluation of effects of drugs, \( S_n/S_1 \) ratios obtained in the presence of antagonists were expressed as percentage of the average of the corresponding \( S_n/S_1 \) control value (see Methods). Values are means ± S.E.M. from 4 – 6 tissue preparations. Significant differences from respective control (solvent): * \( P < 0.05 \) and ** \( P < 0.01 \).

Figure 6. Effects of apyrase and of the ectonucleotidase inhibitor ARL 67156 on evoked tritium overflow from prostatic portion of rat vas deferens in the presence of the P1-antagonists, DPCPX and ZM 241385 and of the P2Y-antagonist RB2. ARL 67156 was tested in the absence (strived column) and in the presence of the P2X-antagonist PPNDS (3 \( \mu \)M; black column). Tissue preparations were incubated with \[^{3}H\]noradrenaline for 40 min and electrically stimulated with 3 trains of 100 pulses/8Hz (S0 – S2). DPCPX (100 nM), ZM 241385 (100 nM) and RB2 (10 \( \mu \)M) were added at the beginning of superfusion and kept throughout. Apyrase (5 U/ml) and ARL 67156 (50 \( \mu \)M) were added 15 min before S2 and removed 10 min after onset of the respective stimulation period. PPNDS (3 \( \mu \)M) was added 20 min before S2 and kept throughout. For evaluation of effects of drugs, \( S_2/S_1 \) ratios obtained in the presence of apyrase or ARL 67156 were expressed as percentage of
the average of the corresponding $S_2/S_1$ control value (see Methods). Values are means ± S.E.M. from 4 – 6 tissue preparations. Significant differences from respective control: * $P < 0.05$ and ** $P < 0.01$; from the effect of ARL 67156 alone, ++ $P < 0.01$. 
Table 1. Basal tritium outflow ($b_1$) and electrically evoked tritium overflow ($S_1$) from prostatic portions of rat vas deferens.

<table>
<thead>
<tr>
<th>Drugs throughout</th>
<th>Basal outflow ($b_1$) (% of tissue tritium.min$^{-1}$)</th>
<th>Evoked overflow ($S_1$) (% of tissue tritium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
<td>0.131 ± 0.008 (14)</td>
<td>1.118 ± 0.093 (14)</td>
</tr>
<tr>
<td>DPCPX (100 nM)</td>
<td>0.140 ± 0.004 (54)</td>
<td>1.120 ± 0.044 (54)</td>
</tr>
<tr>
<td>+ ZM 241385 (100 nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPCPX (100 nM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ ZM 241385 (100 nM)</td>
<td>0.160 ± 0.005 (28) **</td>
<td>1.243 ± 0.090 (28) **</td>
</tr>
<tr>
<td>+ RB2 (10 µM)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Tissue preparations were incubated with $[^3]$H-noradrenaline for 40 min and superfused with medium containing the drugs indicated (in addition to 400 nM desipramine and 1 µM yohimbine that were always present throughout superfusion). $S_1$ was applied after 60 min of superfusion and consisted of 100 pulses/8 Hz; $b_1$ refers to the 5-min period immediately before $S_1$. Values are means ± S.E.M. for ($n$) control preparations. Significant differences from $b_1$ and $S_1$ in the absence of RB2: ** $P < 0.01$. 
Table 2. Effects of P2-antagonists on electrically evoked tritium overflow from prostatic portion of rat vas deferens.

<table>
<thead>
<tr>
<th>Drug added after S₁</th>
<th>µM</th>
<th>(Sₙ/S₁, % of control)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suramin</td>
<td>10</td>
<td>109 ± 6 **</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>120 ± 6 **</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>131 ± 9 **</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>140 ± 5 **</td>
<td>6</td>
</tr>
<tr>
<td>RB2</td>
<td>3</td>
<td>122 ± 5 *</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>139 ± 6 **</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>126 ± 6 *</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>86 ± 7</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>37 ± 4 **</td>
<td>6</td>
</tr>
<tr>
<td>PPADS</td>
<td>3</td>
<td>87 ± 2 *</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>65 ± 2 **</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>29 ± 3 **</td>
<td>9</td>
</tr>
<tr>
<td>PPNDS</td>
<td>1</td>
<td>93 ± 2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>78 ± 3 **</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>39 ± 2 **</td>
<td>6</td>
</tr>
</tbody>
</table>

Tissue preparations were incubated with [³H]-noradrenaline for 40 min and electrically stimulated with 5 trains of 100 pulses/8Hz (S₀ – S₄). P2-antagonists were added 20 min before S₂, S₃ and S₄ at increasing concentrations. For evaluation of the effects of drugs on the electrically evoked tritium overflow, Sₙ/S₁ ratios obtained in the
presence of P2-antagonists were expressed as percentage of the corresponding average control S
/S
 value. Values are means ± S.E.M. from (n) experiments. Significant differences from respective control: * P < 0.05 and ** P < 0.01.
Figure 1. Queiroz et al.

- α,β-meATP
- β,γ-meATP
- β,γ-imidoATP

Tritium overflow (% of control)

P2 Agonists (μM)
Figure 2. Queiroz et al.

![Graph showing the effect of different compounds on Tritium overflow compared to solvent control.](image-url)

- **Solvent**
- **RB2 (10 μM)**
- **2-MeSAMP (10 μM)**
- **MRS 2179 (10 μM)**

Tritium overflow (% of control) vs. 2-MeSATP (μM).
Figure 3. Queiroz et al.  

The graph shows the tritium overflow (% of control) in response to various P2 agonists. The y-axis represents tritium overflow, and the x-axis represents P2 Agonists (µM). The graph includes different concentrations of the following agonists: α,β-meATP, β,γ-meATP, 2-MeSADP, and ATP. The data points indicate significant differences at certain concentrations, as indicated by asterisks (** for p < 0.01 and * for p < 0.05).
Figure 4. Queiroz et al.

**α,β-meATP**

**α,β-meATP + PPNDS (3 μM)**

**β,γ-imidoATP**

**β,γ-meATP + PPNDS (3 μM)**

Tritium overflow (% of control)

80 100 120 140 160

1 10 100

P2 Agonists (μM)
Figure 5. Queiroz et al.

The graph shows the tritium overflow (%) of control for five different P2 antagonists: RB2, 2-MeSAMP, MRS 2179, PPNDS, and NF 279. The x-axis represents P2 Antagonists (µM), and the y-axis represents tritium overflow (% of control). The data points are marked with asterisks indicating statistical significance.
Figure 6. Queiroz et al.

Tritium overflow (% of control)

Apyrase (5 U/ml) + - - -
ARL 67156 (50 μM) - + + +
PPNDS (3 μM) - - + +