British Journal of Pharmacology (1995) 115, 247-254

P_2-purinoceptor-mediated inhibition of noradrenaline release in rat atria

[1]Ivar von Kügelgen, Daniel Stoffel & Klaus Starke

Pharmakologisches Institut, Universität Freiburg, Hermann-Herder-Strasse 5, D79104 Freiburg i. Br., Germany

1 We looked for P_2-purinoceptors modulating noradrenaline release in rat heart atria. Segments of the atria were preincubated with [^{3}H]-noradrenaline and then superfused with medium containing desipramine (1 μM) and yohimbine (1 μM) and stimulated electrically, by 30 pulses/1 Hz unless stated otherwise.

2 The adenosine A_1-receptor agonist, N^6-cyclopentyl-adenosine (CPA; EC_{50} 9.7 nM) and the nucleotides, ATP (EC_{50} 6.6 μM) and adenosine-5'-O-(3-thiotriphosphate) (ATPγS; EC_{50} 4.8 μM), decreased the evoked overflow of tritium. The adenosine A_{2a}-agonist, 2-*p*-(2-carbonylethyl)-phenethylamino-5'-N-ethylcarboxamido-adenosine (CGS-21680; 0.03–0.3 μM) and the P_{2X}-purinoceptor agonist β,γ-methylene-L-ATP (30 μM) caused no change.

3 The concentration-response curve of CPA was shifted to the right by the adenosine A_1-receptor antagonist, 8-cyclopentyl-1,3-dipropyl-xanthine (DPCPX; 3 nM; apparent pK_B value 9.7) but hardly affected by the P_2-purinoceptor antagonist, cibacron blue 3GA (30 μM). In contrast, the concentration-response curves of ATP and ATPγS were shifted to the right by DPCPX (3 nM; apparent pK_B values 9.3 and 9.4, respectively) as well as by cibacron blue 3GA (30 μM; apparent pK_B values 5.0 and 5.1, respectively). Combined administration of DPCPX and cibacron blue 3GA caused a much greater shift of the concentration-response curve of ATP than either antagonist alone. The concentration-response curve of ATP was not changed by indomethacin, atropine or the 5'-nucleotidase blocker α,β-methylene-ADP.

4 Cibacron blue 3GA (30 μM) increased the evoked overflow of tritium by about 70%. The increase was smaller when the slices were stimulated by 9 pulses/100 Hz instead of 30 pulses/1 Hz.

5 The results indicate that the postganglionic sympathetic axons in rat atria possess P_2-purinoceptors in addition to the known adenosine A_1-receptor. Both mediate inhibition of noradrenaline release. Some adenine nucleotides such as ATP and ATPγS act at both receptors. The presynaptic P_2-purinoceptor seems to be activated by an endogenous ligand, presumably ATP, under the condition of these experiments. This is the first evidence for presynaptic P_2-purinoceptors at cardiac postganglionic sympathetic axons.

Keywords: Rat heart atrium; P_1-purinoceptor; P_2-purinoceptor; presynaptic purinoceptors; noradrenaline release; adenine nucleotides; ATP; adenosine-5'-O-(3-thiotriphosphate) (ATPγS); cibacron blue 3GA; suramin

Introduction

Among other locations, P_2-purinoceptors occur presynaptically at terminal noradrenergic axons. Activation of presynaptic P_2-purinoceptors of various types has been reported to *increase* the release of noradrenaline in rabbit ear artery (Miyahara & Suzuki, 1987), guinea-pig ileum (Sperlagh & Vizi, 1991), and chick cultured sympathetic neurones (Allgaier *et al.*, 1994). In mouse and rat vas deferens, rat iris and rat brain cortex, activation of presynaptic P_{2Y}-like purinoceptors *decreases* the release of noradrenaline (von Kügelgen *et al.*, 1989; 1993; 1994b,c; Fuder & Muth, 1993; Kurz *et al.*, 1993). Chick cultured sympathetic neurones possess release-inhibiting P_2-purinoceptors, not classified further, in addition to the release-enhancing receptors (Allgaier *et al.*, 1994). Some peripheral release-inhibiting P_2-purinoceptors seem to function as autoreceptors, i.e. to be activated by endogenous ATP released as postganglionic sympathetic co-transmitter of noradrenaline (Fujioka & Cheung, 1987; Fuder & Muth, 1993; Kurz *et al.*, 1993; von Kügelgen *et al.*, 1993; 1994a,b; Gonçalves & Queiroz, 1994; Grimm *et al.*, 1994).

Adenine nucleotides such as ATP exert P_2-purinoceptor-mediated effects on the heart; the force of contraction, for example, is increased (for review see Ralevic & Burnstock, 1991). We have now looked for presynaptic P_2-purinoceptors at the sympathetic axons innervating rat atria. Adenine nucleosides (Wakade & Wakade, 1978; Khan & Malik, 1980; Richardt *et al.*, 1987) and nucleotides (Khan & Malik, 1980) reduce the release of noradrenaline in the rat heart. The nucleosides act through adenosine A_1-receptors (Richardt *et al.*, 1987). The possibility of an involvement of P_2-purinoceptors in the effect of adenine nucleotides (Khan & Malik, 1980) was not (and at that time could not be) examined. Some results have been published in abstract form (Stoffel *et al.*, 1994).

Methods

Male Wistar rats weighing 240–300 g (Savo, Kisslegg, Germany) were killed by cervical dislocation and exsanguination. The atria were cut into about 14 segments of 4–6 mg (from two rats). Six atrial segments were preincubated at 37°C for 30 min in each of two vials containing 4 ml medium with (−)-[^{3}H]-noradrenaline (0.1 μM). The segments were then washed three times with [^{3}H]-noradrenaline-free medium. One segment was transferred to each of twelve superfusion chambers where it was held by a polypropylene mesh between platinum plate electrodes 4 mm apart. The tissue was superfused with [^{3}H]-noradrenaline-free medium for 144 min at 1 ml min^{-1} and 37°C. A Stimulator I (Hugo Sachs Elektronik, March-Hugstetten, Germany) operating in the constant current mode was used for electrical field stimulation. Five periods of stimulation were applied (rectangular

[1] Author for correspondence.

pulses of 1 ms width and 60 mA current strength). The first, delivered after 30 min of superfusion and consisting of 30 pulses/1 Hz, was not used for determination of tritium overflow. The following stimulation periods (S_1–S_4) began after 66, 87, 108 and 129 min of superfusion and consisted of 30 pulses/1 Hz unless stated otherwise. The collection of successive 3-min superfusate samples began 6 min before S_1. After superfusion, each tissue segment was solubilized, and tritium was measured in superfusate samples and solubilized tissues by liquid scintillation counting.

The superfusion medium contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 0.9, glucose 11, ascorbic acid 0.3 and disodium EDTA 0.03. The medium used for preincubation contained $CaCl_2$ 0.2 mM instead of 2.5 mM (see Limberger *et al.*, 1992). Media were saturated with 5% CO_2 in O_2. The pH was adjusted to 7.4 with NaOH 1 M. The superfusion but not the preincubation medium contained in addition, desipramine (1 μM) and, in most experiments, yohimbine (1 μM) in order to block uptake$_1$ and presynaptic α_2-adrenoceptors, respectively. Other drugs were present either throughout superfusion, or from 6 min before S_2 for the remainder of the experiment, or, at increasing concentrations, from 6 min before to 15 min after the onset of S_2, S_3 and S_4. The delay from addition of drug to medium to arrival at tissue was about 60 s.

The outflow of tritium was expressed as fractional rate (min^{-1}) (Kurz *et al.*, 1993). The electrically evoked overflow was calculated as the difference 'total overflow during the 9 min after onset of stimulation' minus 'estimated basal outflow'; the basal outflow was assumed to decline linearly from the 3-min interval before, to the interval 9–12 min after, onset of stimulation. The difference (total minus basal; Bq) was expressed as a percentage of the tritium content (Bq) of the tissue at the onset of stimulation. Effects of drugs that were added after S_1 on basal tritium efflux were evaluated as ratios of the fractional rate immediately before S_2, S_3 and S_4 and the fractional rate immediately before S_1 (b_n/b_1). Effects of drugs that were added after S_1 on electrically evoked overflow were evaluated as ratios of the overflow elicited by S_2, S_3 and S_4 and the overflow elicited by S_1 (S_n/S_1). S_n/S_1 ratios obtained in individual experiments in which a test compound A was added after S_1 were calculated as a percentage of the respective mean ratio in the appropriate control group (solvent instead of A). When the interaction of A, added after S_1, and a drug B, added throughout superfusion, was studied, the 'appropriate control' was a group in which B alone was used.

Where relevant, the sigmoid-shaped function No. 25 of Waud (1976) was fitted to averaged agonist concentration-inhibition data. The function yielded the maximal inhibition and the EC_{50} (concentration that caused 50% of the maximal inhibition). For concentration-inhibition data from experiments carried out in the presence of the antagonists DPCPX or cibacron blue 3GA, the maximal inhibition was fixed to that obtained in the absence of antagonist (cf. Kurz *et al.*, 1993). pK_B ($-\log K_B$) values of DPCPX and cibacron blue 3GA were calculated from the increase in EC_{50} values. Since only one antagonist concentration was used and a competitive character of the antagonism was not verified, the values are *apparent* pK_B values (cf. von Kügelgen *et al.*, 1994c).

Drugs used were: suramin hexasodium (Bayer, Wuppertal, Germany), (−)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 1.48–2.65 TBq $mmol^{-1}$ (Du Pont, Dreieich, Germany), atropine sulphate (Merck, Darmstadt, Germany), 2-*p*-(2-carbonylethyl)-phenethylamino-5′-N-ethylcarboxamido-adenosine HCl (CGS-21680), N^6-cyclopentyl-adenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), β,γ-methylene-L-adenosine-5′-triphosphate tetrasodium (β,γ-methylene-L-ATP), (−)-propranolol HCl (Research Biochemicals, Biotrend, Köln, Germany), yohimbine HCl (Roth, Karlsruhe, Germany), adenosine-5′-O-(3-thiotriphosphate) tetralithium (ATPγS), ATP disodium, cibacron blue 3GA (C-9534 in Sigma catalogue 1994; isomer of reactive blue 2 in which the sulphonic acid residue at the terminal benzene ring is in the *o*-position; see footnote on p. 130 of von Kügelgen *et al.*, 1994b), desipramine HCl, indomethacin, α,β-methylene-adenosine-5′-diphosphate (α,β-methylene-ADP), tetrodotoxin (Sigma, Deisenhofen, Germany). Solutions of drugs were prepared with either distilled water, or (indomethacin) the KH_2PO_4- and $NaHCO_3$-containing stock solution of the medium, or (CGS-21680, DPCPX) dimethyl sulphoxide (final concentration about 0.1 mM), or (CPA) ethanol (final concentration about 1 mM), or (tetrodotoxin) sodium acetate buffer (0.1 M, pH 4.8). The solvents did not change basal tritium efflux or the evoked overflow. Dimethyl sulphoxide (0.1 mM) was added in all experiments throughout superfusion to make them directly comparable.

Means ± s.e.mean are given throughout. Differences between means were tested for significance by the Mann-Whitney test. $P<0.05$ or lower was taken as the criterion of statistical significance. For multiple comparisons with the same control, P levels were adjusted according to Bonferroni. n is the number of tissue pieces.

Results

Stimulation by 30 pulses/1 Hz markedly increased the outflow of tritium from atrial segments preincubated with [^{3}H]-noradrenaline (Figure 1a). When the superfusion medium contained yohimbine (1 μM) in addition to desipramine (1 μM), as in the majority of experiments, the fractional rate of efflux immediately before S_1 (b_1) averaged 0.00161 ± 0.00003 min^{-1}, corresponding to 21.6 ± 0.6 Bq min^{-1}, and the overflow at S_1 (Table 1) 1.025 ± 0.037% of the tritium content of the tissue, corresponding to 150.7 ± 8.4 Bq (n = 132). Experimentally induced changes will be mentioned below.

When solvent was administered after S_1 (6 min before S_2), the b_2/b_1, b_3/b_1, and b_4/b_1 ratios were 0.97 ± 0.01, 0.97 ± 0.01 and 0.94 ± 0.02, and S_2/S_1, S_3/S_1, and S_4/S_1 ratios 1.01 ± 0.01, 0.99 ± 0.01 and 0.98 ± 0.01, respectively (n = 12; Figure 1a). Average b_n/b_1 ratios also were slightly below unity, and S_n/S_1 ratios close to unity, when the additional compounds listed in Table 1 were present in the medium throughout superfusion and solvent was added after S_1 (not shown).

Evoked tritium overflow: adenine nucleosides and nucleotides

In an initial series of experiments, drugs were added after S_1 and then kept at a constant concentration. Tetrodotoxin (0.3 μM) abolished the evoked overflow of tritium (Figure 2a). The adenosine A_1-receptor agonist CPA (0.3 μM; Williams *et al.*, 1986) as well as the nucleotides ATP (30 and 300 μM) and ATPγS (30 μM), a metabolically more stable analogue (Welford *et al.*, 1986), caused marked inhibition which was approximately constant from S_2, after 6 min of exposure, to S_4, after 48 min of exposure (Figure 2). No change was observed with the adenosine A_{2a}-agonist, CGS-21680 (0.03 and 0.3 μM; Jarvis *et al.*, 1989; Figure 2a) and the metabolically stable P_{2X}-selective ATP analogue β,γ-methylene-L-ATP (30 μM; Hourani *et al.*, 1986; Figure 2c).

When added after S_1 at increasing concentrations, CPA, ATP and ATPγS reduced the evoked overflow of tritium in a concentration-dependent manner (ATP in Figure 1b; concentration-inhibition curves in Figures 3 to 5, open symbols). The EC_{50} values (maximal inhibitions) were 9.7 nM (89%) for CPA, 6.6 μM (95%) for ATP, and 4.8 μM (88%) for ATPγS.

Evoked tritium overflow: interactions

Drugs tested for their interaction with CPA, ATP and ATPγS were added throughout superfusion (in addition to desipramine and yohimbine). When thus applied, the

adenosine A_1-receptor antagonist, DPCPX (3 nM; Bruns *et al.*, 1987; Lohse *et al.*, 1987) slightly increased the overflow evoked by S_1 (Table 1). It shifted the concentration-inhibition curves of CPA, ATP and ATPγS to the right by similar degrees (Figure 3; solid symbols). The shifts correspond to apparent pK_B values of DPCPX against CPA, ATP and ATPγS of 9.7, 9.3 and 9.4, respectively.

The P_2-purinoceptor antagonist, cibacron blue 3GA (30 μM; Kerr & Krantis, 1979; Fuder & Muth, 1993; see footnote in von Kügelgen *et al.*, 1994b) increased the

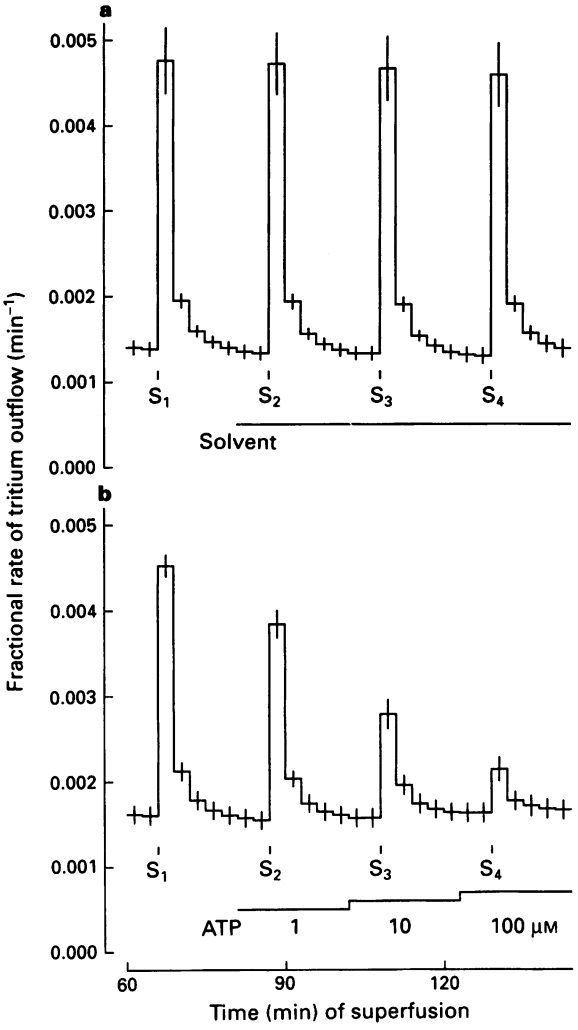

Figure 1 Time course of tritium outflow from segments of rat atria and effect of ATP. After preincubation with [^{3}H]-noradrenaline, tissue segments were superfused with medium containing desipramine (1 μM) and yohimbine (1 μM). They were stimulated four times by 30 pulses/1 Hz (S_1–S_4). Solvent (a; $n = 12$) or ATP (b; $n = 10$) was added as indicated.

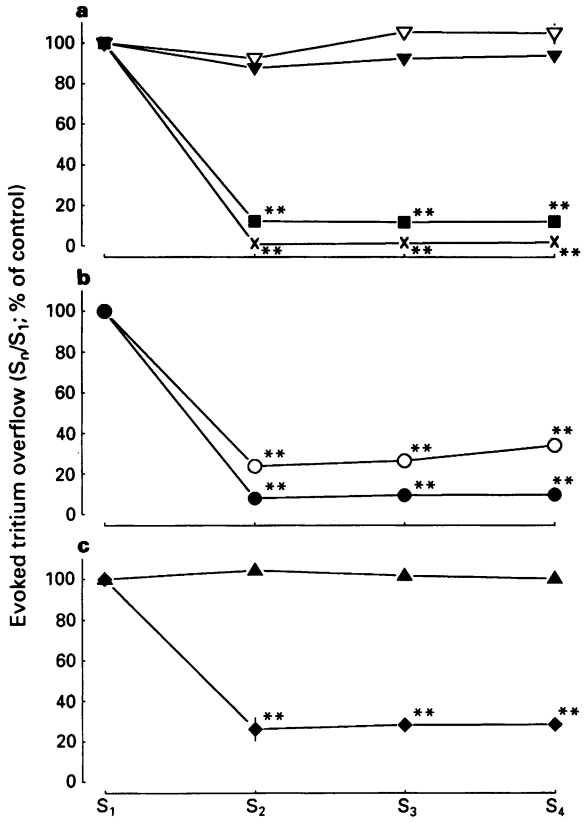

Figure 2 Effects of purinoceptor agonists and tetrodotoxin on electrically evoked overflow of tritium. After preincubation with [^{3}H]-noradrenaline, atrial segments were superfused with medium containing desipramine (1 μM) and yohimbine (1 μM). They were stimulated four times by 30 pulses/1 Hz (S_1–S_4). 2-*p*-(2-Carbonyethyl)-phenethylamino-5′-N-ethylcarboxamido-adenosine (CGS-21680; ▽ 0.03 and ▼ 0.3 μM; a), N^6-cyclopentyl-adenosine (CPA; ■ 0.3 μM; a), tetrodotoxin (×, 0.3 μM; a), ATP (○ 30 and ● 300 μM; b), β,γ-methylene-L-ATP (▲ 30 μM; c) or adenosine-5′-O-(3-thiotriphosphate) (ATPγS; ◆ 30 μM; c) was added 6 min before S_2 for the remainder of the experiment. Ordinates, evoked tritium overflow: S_n/S_1 ratios obtained in individual tissue segments were calculated as percentage of the corresponding average control S_n/S_1 ratio. Means ± s.e.mean from 4–11 tissue segments. Significant differences from corresponding control: $^{**}P < 0.01$.

Table 1 Electrically evoked tritium overflow (S_1)

Drugs present throughout superfusion	*Overflow evoked by S_1* (% of tissue tritium)	n
–	1.025 ± 0.037	132
DPCPX 3 nM	1.272 ± 0.046**	48
Cibacron blue 3GA 30 μM	1.914 ± 0.057**	43
Cibacron blue 3GA 30 μM[a]	1.465 ± 0.151*	24
DPCPX 3 nM + cibacron blue 3GA 30 μM	2.142 ± 0.135**	16
α,β-Methylene-ADP 100 μM	1.228 ± 0.125	11
Indomethacin 10 μM	1.263 ± 0.082*	24
Indomethacin 10 μM + cibacron blue 3GA 30 μM	1.795 ± 0.090**	24
Atropine 1 μM	1.631 ± 0.097**	20
Indomethacin 10 μM + atropine 1 μM + propranolol 1 μM + DPCPX 3 nM	1.359 ± 0.071**	12

After preincubation with [^{3}H]-noradrenaline, atrial segments were superfused with medium containing the drugs indicated (in addition to desipramine, 1 μM, and yohimbine, 1 μM, which were also present throughout superfusion). S_1 was applied after 66 min of superfusion and consisted of 30 pulses/1 Hz. DPCPX, 8-cyclopentyl-1,3-dipropylxanthine. Means ± s.e.mean from *n* tissue segments.
[a]Current strength 30 instead of 60 mA.
Significant differences from experiments, shown in first line, in which only desipramine and yohimbine were present: $^*P < 0.05$ and $^{**}P < 0.01$.

overflow at S_1 by 87% (Table 1). It caused little if any change in the concentration-response curve of CPA but shifted the curves of ATP and ATPγS clearly to the right (Figure 4; solid symbols). Apparent pK_B values of cibacron blue 3GA against ATP and ATPγS were 5.0 and 5.1, respectively.

An increase in transmitter release may *per se* attenuates the modulation of release through presynaptic receptors, irrespective of the cause of the increase (see p. 926 of Starke *et al.*, 1989). Therefore, in some experiments with cibacron blue 3GA (30 μM) the current strength was lowered from 60 to 30 mA in order to bring the reference overflow, at S_1, in the presence of cibacron blue 3GA, closer to values obtained in the absence of the antagonist (Table 1). Cibacron blue 3GA 30 μM shifted the concentration-inhibition curve of ATP to the right despite the S_1 adjustment (compare Figure 4b and 4d), indicating that the shift was in fact due to blockade of P_2-purinoceptors rather than to the increase in transmitter release *per se* (see von Kügelgen *et al.*, 1992). [We did not determine a concentration-inhibition curve of ATP at 30 mA in the absence of cibacron blue 3GA; from all that is known on presynaptic receptors it would lie to the left of the curve determined at 60 mA (p. 102 of Starke, 1987), so the shift by cibacron blue 3GA would be even more pronounced than shown in Figure 4d.]

Combined administration of cibacron blue 3GA (30 μM) and DPCPX (3 nM) enhanced the overflow evoked by S_1 by 109% (Table 1) and shifted the concentration-response curve of ATP beyond the shifts caused by DPCPX alone (Figure 3b) and cibacron blue 3GA alone (Figure 4b). The shift beyond that produced by DPCPX alone (Figure 3b) corresponds to an apparent pK_B of cibacron blue 3GA against ATP of 4.7, close to the 5.0 obtained in the absence of DPCPX (Figure 4b).

Further interaction experiments were carried out in search for possible mediators of the inhibitory effect of ATP. α,β-Methylene-ADP (100 μM) was used to block 5'-nucleotidase (Fredholm *et al.*, 1982; Fleetwood & Gordon, 1987; Borst & Schrader, 1991), the enzyme that catalyses the dephosphorylation of AMP to adenosine. α,β-Methylene-ADP did not change S_1 (Table 1) and did not affect the concentration-response curve of ATP (Figure 5a). Indomethacin (10 μM) and atropine (1 μM) were used at concentrations known to block the synthesis of prostaglandins in the heart (Starke *et al.*, 1977; Khan & Malik, 1980) and presynaptic muscarinic receptors (Fozard & Muscholl, 1972), respectively. Both increased the overflow at S_1 (Table 1), possibly by removing a presynaptic inhibition by prostaglandins and acetylcholine (see Fuder & Muscholl, 1995). Neither indomethacin (Figure

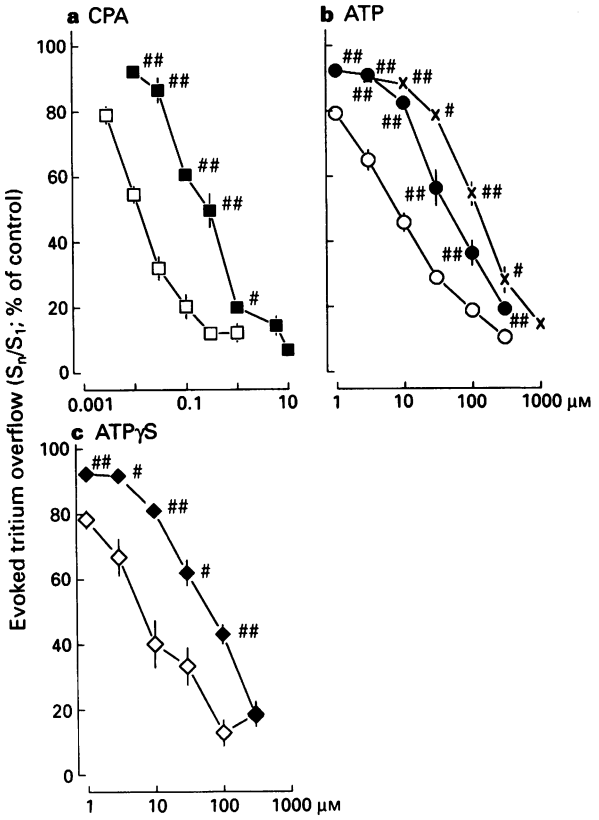

Figure 3 Interaction of purinoceptor agonists with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or cibacron blue 3GA combined with DPCPX. After preincubation with [^{3}H]-noradrenaline, atrial segments were superfused with medium containing desipramine (1 μM) and yohimbine (1 μM). They were stimulated four times by 30 pulses/1 Hz (S_1–S_4). N^6-cyclopentyl-adenosine (CPA, a), ATP (b) or adenosine-5'-O-(3-thiotriphosphate) (ATPγS, c) was added at increasing concentrations from 6 min before to 15 min after onset of S_2, S_3 and S_4. Open symbols, experiments in which CPA, ATP or ATPγS was given alone; solid symbols, experiments in which medium contained DPCPX (3 nM) throughout superfusion; (×) experiments in which medium contained both DPCPX (3 nM) and cibacron blue 3GA (30 μM) throughout superfusion (b). Ordinates, evoked tritium overflow: S_n/S_1 ratios obtained in individual tissue segments were calculated as percentage of the corresponding average control S_n/S_1 ratio. Means ± s.e.mean from 4–10 tissue segments. Significant differences from experiments in which CPA, ATP or ATPγS was given alone: $^{\#}P<0.05$ and $^{\#\#}P<0.01$.

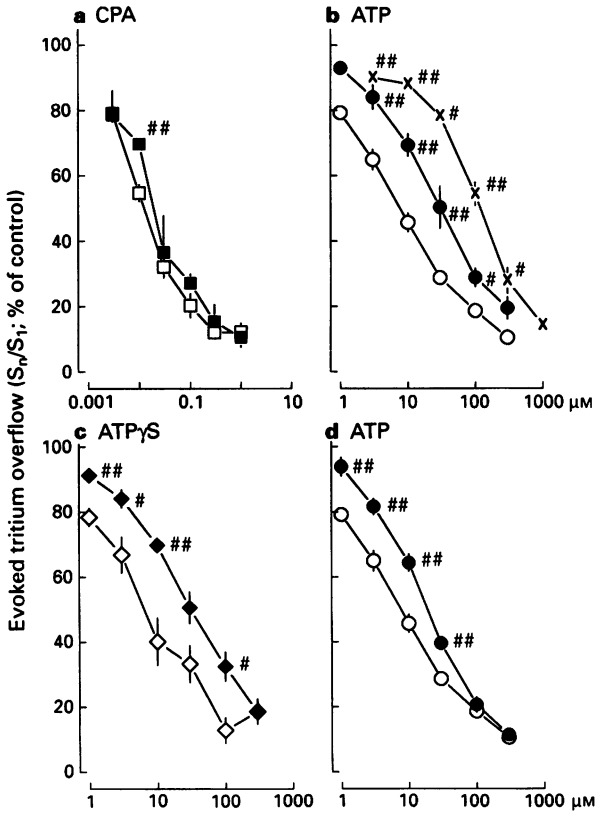

Figure 4 Interaction of purinoceptor agonists with cibacron blue 3GA or 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) combined with cibacron blue 3GA. Open symbols, experiments in which N^6-cyclopentyl-adenosine (CPA, a), ATP (b and d) or adenosine-5'-O-(3-thiotriphosphate) (ATPγS, c) was given alone (identical with Figure 3); solid symbols, experiments in which medium contained cibacron blue 3GA (30 μM) throughout superfusion; (×) experiments in which medium contained both cibacron blue 3GA (30 μM) and DPCPX (3 nM) throughout superfusion (b; identical with (×) in Figure 3b). In some experiments in which cibacron blue 3GA was tested against ATP the current strength for electrical stimulation was reduced from 60 to 30 mA (solid symbols in d). Means ± s.e.mean from 4–11 tissue segments. Other details as in Figure 3.

5b) nor atropine (Figure 5c) changed the concentration-response curve of ATP. Cibacron blue 3GA (30 μM) shifted the concentration-response curve of ATP to the right also in the presence of indomethacin (10 μM; Figure 5b). The combination of indomethacin and cibacron blue 3GA increased the overflow of tritium at S_1 as did each compound alone (Table 1).

Evoked tritium overflow: purinoceptor antagonists

Effects of purinoceptor antagonists on the evoked overflow of tritium were already observed when they were present throughout superfusion (S_1; Table 1). However, drug effects are better assessed in this kind of experiment when the drugs are given after S_1 so that S_1 is the reference for each tissue segment (von Kügelgen *et al.*, 1994b). Cibacron blue 3GA 30 μM, when thus administered in desipramine- and yohimbine-containing medium, increased the overflow of tritium evoked by 30 pulses/1 Hz by 67% (Table 2), similar to the 87% increase of S_1 when cibacron blue 3GA was applied throughout superfusion (Table 1). The P_2-purinoceptor antagonist, suramin (300 μM; Dunn & Blakeley, 1988) increased the evoked overflow by 9% only (Table 2). DPCPX (3 nM) caused no change (Table 2), and this questions the relevance of the slight increases in S_1 observed when DPCPX was present throughout superfusion.

Cibacron blue 3GA 30 μM increased the overflow of tritium evoked by 30 pulses/1 Hz to a similar extent, namely by 72.6 ± 7.4%, when the medium contained indomethacin (10 μM), atropine (1 μM), propranolol (1 μM) and DPCPX (3 nM) in addition to desipramine and yohimbine throughout superfusion ($n = 6$; protocol of Table 2; S_1 in Table 1).

Cibacron blue 3GA also increased the tritium overflow evoked by 30 pulses/1 Hz, and DPCPX also failed to cause a significant change, when yohimbine was omitted from the medium (Table 2). In these experiments the overflow at S_1 was 0.316 ± 0.019% of the tritium content of the tissue ($n = 24$), about one third of that observed in presence of desipramine and yohimbine (Table 1). Accordingly, yohimbine (1 μM) increased the overflow evoked by 30 pulses/1 Hz about threefold when added after S_1 to previously yohimbine-free medium (Table 2).

Finally, trains consisting of 9 pulses/100 Hz were used for stimulation (Table 2). The overflow of tritium elicited by S_1 was 0.217 ± 0.014% of tissue tritium ($n = 24$). DPCPX and yohimbine did not affect the evoked overflow. Cibacron blue 3GA caused an increase by 38%, significantly ($P < 0.01$) less than in slices stimulated by trains of 30 pulses/1 Hz (Table 2).

Basal tritium efflux

None of the drugs, whether present throughout superfusion (in addition to desipramine) or added after S_1, changed significantly the basal efflux of tritium, with two exceptions: ATP (100 μM) caused a slight increase (see Figure 1b), and DPCPX (3 nM), when present throughout superfusion, caused a small decrease (not shown).

Discussion

The electrically evoked overflow of tritium in experiments of this kind reflects action potential-induced, neural release of [^{3}H]-noradrenaline (cf. for rat heart Fuder *et al.*, 1982). The

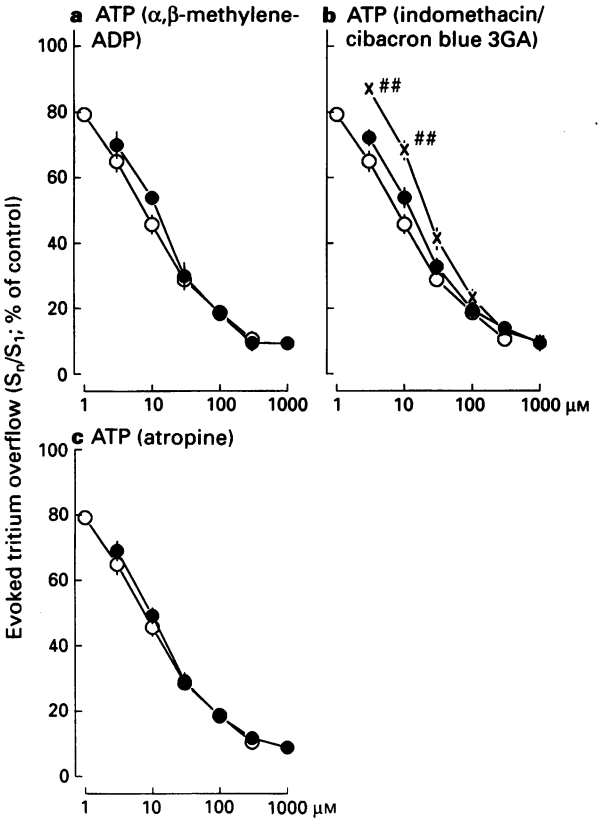

Figure 5 Interaction of ATP with α,β-methylene-ADP, indomethacin or indomethacin combined with cibacron blue 3GA, and atropine. Open symbols, experiments in which ATP was given alone (identical with Figure 3); solid symbols, experiments in which medium contained α,β-methylene-ADP (100 μM; a), indomethacin (10 μM; b) or atropine (1 μM; c) throughout superfusion; (×) experiments in which medium contained both indomethacin (10 μM) and cibacron blue 3GA (30 μM) throughout superfusion (b). Means ± s.e.mean from 4–11 tissue segments. Other details as in Figure 3.

Table 2 Effects of purinoceptor antagonists and yohimbine on electrically evoked tritium overflow

Drugs added 6 min before S_2	*Evoked tritium overflow* (S_2 to S_4/S_1; % of control) 30 p/1 Hz Yohimbine (1 μM) present	30 p/1 Hz No yohimbine	9 p/100 Hz No yohimbine
–	100.0 ± 0.9 (6)	100.0 ± 2.9 (6)	100.0 ± 5.0 (6)
DPCPX 3 nM	103.9 ± 3.8 (5)	107.8 ± 3.9 (6)	108.5 ± 6.0 (6)
Cibacron blue 3GA 30 μM	166.9 ± 5.1 (5)*	191.2 ± 11.5 (6)**	138.0 ± 9.5 (6)*
Suramin 300 μM	108.6 ± 1.7 (6)**	–	–
Yohimbine 1 μM	–	326.1 ± 23.1 (6)**	110.7 ± 4.5 (5)

After preincubation with [^{3}H]-noradrenaline, atrial segments were superfused with medium containing desipramine (1 μM) and, where indicated in heading, yohimbine (1 μM). They were stimulated electrically four times (S_1–S_4) at pulse numbers and frequencies indicated. 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX), cibacron blue 3GA, suramin or yohimbine was added 6 min before S_2 for the remainder of the experiment. Ratios 'average overflow at S_2 to S_4 over overflow at S_1' obtained in individual tissue segments were calculated as a percentage of the corresponding average control ratio. Means ± s.e.mean from (n) tissue segments.
Significant differences from corresponding control (first line): *$P < 0.05$ and **$P < 0.01$.

medium contained desipramine (1 μM) and in most experiments yohimbine (1 μM), thus ensuring that changes in evoked tritium overflow caused by purinoceptor ligands were not due to an interference with uptake$_1$ or α_2-adrenoceptors.

Presynaptic adenosine A_1-receptors

Our experiments confirm the operation of release-inhibiting P_1-purinoceptors of the A_1-subtype at the sympathetic terminal axons in rat heart (see Introduction). In accord with this assumption, only the A_1-receptor agonist, CPA, but not the A_{2a}-receptor agonist, CGS-21680 affected the release of noradrenaline. Moreover, the adenosine A_1-receptor antagonist, DPCPX shifted the concentration-response curve of CPA to the right with an apparent pK_B value (9.7) close to values found at presynaptic A_1-receptors in other rat tissues (9.3–9.8; Sebastião *et al.*, 1990; Fuder *et al.*, 1992; Kurz *et al.*, 1993; von Kügelgen *et al.*, 1994c). The release-inhibiting P_1-purinoceptors at the sympathetic axons of guinea-pig papillary muscles also belong to the A_1-subtype (Schütz *et al.*, 1991).

The presynaptic A_1-receptors also mediated part of the inhibition caused by adenine nucleotides. DPCPX shifted the concentration-response curves of ATP and ATPγS to the right with apparent pK_B values (9.3 and 9.4) similar to the pK_B against CPA (9.7). Blockade of 5'-nucleotidase by α,β-methylene-ADP did not change the inhibition produced by ATP, indicating that breakdown to adenosine was not necessary for the effect. The result supports previous conclusions that some adenine nucleotides activate adenosine (A_1)-receptors directly (noradrenergic axons: von Kügelgen *et al.*, 1992; 1994b,c; Fuder & Muth, 1993; Kurz *et al.*, 1993; non-noradrenergic axons: Moody *et al.*, 1984; Wiklund *et al.*, 1985; Rubino *et al.*, 1992; non-neural cells: Collis & Pettinger, 1982; Bailey *et al.*, 1992). The common presynaptic receptor for adenine nucleosides and nucleotides at the sympathetic fibres of rat tail artery and rabbit vas deferens has been proposed to be a new 'hybrid' P_3-purinoceptor (Shinozuka *et al.*, 1988; Todorov *et al.*, 1994). In rat atria, the identical pK_B values of DPCPX against CPA, ATP and ATPγS (9.3–9.7; see above) indicate that the presynaptic receptor is the classical adenosine A_1-receptor (compare von Kügelgen *et al.*, 1992; 1994c; Kurz *et al.*, 1993).

Presynaptic P_2-purinoceptors

Presynaptic A_1-receptors were not the only site of inhibition by ATP and ATPγS: there was an additional, P_2-purinoceptor, site. The P_2 antagonist, cibacron blue 3GA, hardly changed the effect of CPA, but clearly shifted the concentration-inhibition curves of ATP and ATPγS to the right, with apparent pK_B values (5.0 and 5.1) close to those found at the presynaptic P_2-purinoceptors in rat iris (4.7; Fuder & Muth, 1993) and brain cortex (5.0; von Kügelgen *et al.*, 1994c). Cibacron blue 3GA produced a similar shift (apparent pK_B 4.7) when tested in combination with DPCPX (beyond the shift caused by DPCPX alone), as predicted from theory when there are two different receptors for an agonist. Atropine failed to change the effect of ATP, thus excluding a cholinergic link. Indomethacin also did not alter the effect of ATP (confirming Khan & Malik, 1980), and cibacron blue 3GA shifted the concentration-inhibition curve of ATP even in the presence of indomethacin, although the shift was somewhat reduced; at least the major part of the inhibition by ATP, therefore, was independent of cyclo-oxygenase products. In all likelihood, the P_2-purinoceptors are located at the sympathetic terminal axons themselves. Co-existence of presynaptic A_1- and P_2-purinoceptors has also been found in mouse and rat vas deferens, rat iris and rat brain cortex (von Kügelgen *et al.*, 1989; 1994b,c; Fuder & Muth, 1993; Kurz *et al.*, 1993).

The P_2-purinoceptors at the noradrenergic axons of mouse and rat vas deferens, rat iris and rat brain cortex are P_{2Y}-like (von Kügelgen *et al.*, 1989; 1993; 1994b,c; Fuder & Muth, 1993; Kurz *et al.*, 1993). The lack of any effect of the highly selective P_{2x}-purinoceptor agonist β,γ-methylene-L-ATP and the similarity of the pK_B values of cibacron blue 3GA in rat atria, iris and brain cortex (see above) suggest the same for rat atria.

Endogenous input

The P_2-purinoceptors at the postganglionic sympathetic axons innervating mouse, rat and rabbit vas deferens, rat iris, rat tail artery and guinea-pig saphenous artery seem to be autoreceptors, i.e. to be activated by an endogenous ligand, presumably ATP, released as cotransmitter of noradrenaline (see Introduction). The present results suggest the same for rat atria: cibacron blue 3GA and, although to a much smaller extent, suramin, but not (or not consistently) DPCPX, increased the release of [^{3}H]-noradrenaline. The increase by cibacron blue 3GA was observed in the combined presence of desipramine, yohimbine, atropine, DPCPX, indomethacin and propranolol, thus excluding the uptake$_1$ carrier, α_2-adrenoceptors, muscarinic receptors, adenosine A_1-receptors, cyclo-oxygenase and β-adrenoceptors as potential sites of, or links in, the action of cibacron blue 3GA. Also in accord with an autoreceptor role is the dependence of the increase on the stimulation conditions: it was larger for relatively long (30 pulses/1 Hz) than for very short pulse trains (9 pulses/100 Hz); similar conditions of operation have been established for the P_2-autoreceptors in mouse and rat vas deferens (von Kügelgen *et al.*, 1993; 1994b) as well as for other presynaptic autoreceptors (Starke *et al.*, 1989; α_2-autoreceptors in rat atria: Limberger *et al.*, 1992).

However, alternative sources of the endogenous agonist have to be taken into consideration. Adenine nucleotides are released from postganglionic parasympathetic in addition to sympathetic axon terminals (see Burnstock, 1990; von Kügelgen & Starke, 1991; Hoyle, 1992; Zimmermann, 1994) as well as from non-neural cells such as, in the heart, cardiomyocytes and endocardial cells (Paddle & Burnstock, 1974; Fredholm *et al.*, 1982; Borst & Schrader, 1991). Antagonism against ATP coming from these cells may have contributed to the increase of [^{3}H]-noradrenaline release caused by cibacron blue 3GA and suramin. Hypoxia greatly increases the release of ATP from cardiac non-neural cells (Paddle & Burnstock, 1974; Borst & Schrader, 1991). Whether responding to cotransmitter ATP, ATP from parasympathetic axons, or ATP of non-neural origin, the P_2-purinoceptors at cardiac sympathetic axon terminals will mediate inhibition of noradrenaline release and therefore, like the presynaptic A_1-receptors (see Richardt *et al.*, 1987), may protect the heart from sympathetic overdrive. Activation of excitatory soma-dendritic P_2-purinoceptors at the cholinergic neurones of the heart (Fieber & Adams, 1991) would act in the same direction.

The study was supported by the Deutsche Forschungsgemeinschaft (SFB 325). We thank Prof. H.J. Ruoff (Bayer, Fachbereich Klinische Forschung, Wuppertal, Germany) and Merck (Darmstadt, Germany) for drugs.

References

ALLGAIER, C., PULLMANN, F., SCHOBERT, A., VON KÜGELGEN, I. & HERTTING, G. (1994). P_2 purinoceptors modulating noradrenaline release from sympathetic neurons in culture. *Eur. J. Pharmacol.*, **252**, R7–R8.

BAILEY, S.J., HICKMAN, D. & HOURANI, S.M.O. (1992). Characterization of P_1-purinoceptors mediating contraction of the rat colon muscularis mucosae. *Br. J. Pharmacol.*, **105**, 400–404.

BORST, M.M. & SCHRADER, J. (1991). Adenine nucleotide release from isolated perfused guinea pig hearts and extracellular formation of adenosine. *Circ. Res.*, **68**, 797–806.

BRUNS, R.F., FERGUS, J.H., BADGER, E.W., BRISTOL, J.A., SANTAY, L.A., HARTMAN, J.D., HAYS, S.J. & HUANG, C.C. (1987). Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-1,3-dipropylxanthine to rat brain membranes. *Naunyn-Schmied. Arch. Pharmacol.*, **335**, 59–63.

BURNSTOCK, G. (1990). Co-transmission. *Arch. Int. Pharmacodyn.*, **304**, 7–33.

COLLIS, M.G. & PETTINGER, S.J. (1982). Can ATP stimulate P_1-receptors in guinea-pig atrium without conversion to adenosine? *Eur. J. Pharmacol.*, **81**, 521–529.

DUNN, P.M. & BLAKELEY, A.G.H. (1988). Suramin: a reversible P_2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.*, **93**, 243–245.

FIEBER, L.A. & ADAMS, D.J. (1991). Adenosine triphosphate-evoked currents in cultured neurones dissociated from rat parasympathetic cardiac ganglia. *J. Physiol.*, **434**, 239–256.

FLEETWOOD, G. & GORDON, J.L. (1987). Purinoceptors in the rat heart. *Br. J. Pharmacol.*, **90**, 219–227.

FOZARD, J.R. & MUSCHOLL, E. (1972). Effects of several muscarinic agonists on cardiac performance and the release of noradrenaline from sympathetic nerves of the perfused rabbit heart. *Br. J. Pharmacol.*, **45**, 616–629.

FREDHOLM, B.B., HEDQVIST, P., LINDSTRÖM, K. & WENNMALM, M. (1982). Release of nucleosides and nucleotides from the rabbit heart by sympathetic nerve stimulation. *Acta Physiol. Scand.*, **116**, 285–295.

FUDER, H., BRINK, A., MEINCKE, M. & TAUBER, U. (1992). Purinoceptor-mediated modulation by endogenous and exogenous agonists of stimulation-evoked [^{3}H]noradrenaline release on rat iris. *Naunyn-Schmied. Arch. Pharmacol.*, **345**, 417–423.

FUDER, H. & MUSCHOLL, E. (1995). Heteroreceptor-mediated modulation of noradrenaline and acetylcholine release from peripheral nerves. *Rev. Physiol. Biochem. Pharmacol.*, **126**, 265–412.

FUDER, H. & MUTH, U. (1993). ATP and endogenous agonists inhibit evoked [^{3}H]-noradrenaline release in rat iris via A_1- and P_{2Y}-like purinoceptors. *Naunyn-Schmied. Arch. Pharmacol.*, **348**, 352–357.

FUDER, H., SIEBENBORN, R. & MUSCHOLL, E. (1982). Nicotine receptors do not modulate the ^{3}H-noradrenaline release from the isolated rat heart evoked by sympathetic nerve stimulation. *Naunyn-Schmied. Arch. Pharmacol.*, **318**, 301–307.

FUJIOKA, M. & CHEUNG, D.W. (1987). Autoregulation of neuromuscular transmission in the guinea-pig saphenous artery. *Eur. J. Pharmacol.*, **139**, 147–153.

GONÇALVES, J. & QUEIROZ, M.G. (1994). Endogenous purines modulate noradrenaline release in the rat tail artery via A_{2A} and P_2 purinoceptors. *Drug Devel. Res.*, **31**, 274.

GRIMM, U., FUDER, H., MOSER, U., BÄUMERT, H.G., MUTSCHLER, E. & LAMBRECHT, G. (1994). Characterization of the prejunctional muscarinic receptors mediating inhibition of evoked release of endogenous noradrenaline in rabbit isolated vas deferens. *Naunyn-Schmied. Arch. Pharmacol.*, **349**, 1–10.

HOURANI, S.M.O., LOIZOU, G.D. & CUSACK, N.J. (1986). Pharmacological effects of L-AMP-PCP on ATP receptors in smooth muscle. *Eur. J. Pharmacol.*, **131**, 99–103.

HOYLE, C.H.V. (1992). Transmission: purines. In *Autonomic Neuroeffector Mechanisms*. ed. Burnstock, G. & Hoyle, C.H.V. pp. 367–407. Chur: Harwood.

JARVIS, M.F., SCHULZ, R., HUTCHISON, A.J., DO, U.H., SILLS, M.A. & WILLIAMS, M. (1989). [^{3}H]CGS 21680, a selective A_2 adenosine receptor agonist directly labels A_2 receptors in rat brain. *J. Pharmacol. Exp. Ther.*, **251**, 888–893.

KERR, D.I.B. & KRANTIS, A. (1979). A new class of ATP antagonist. *Proc. Aust. Physiol. Pharmacol. Soc.*, **10**, 156P.

KHAN, M.T. & MALIK, K.U. (1980). Inhibitory effect of adenosine and adenine nucleotides on potassium-evoked efflux of [^{3}H]-noradrenaline from the rat isolated heart: lack of relationship to prostaglandins. *Br. J. Pharmacol.*, **68**, 551–561.

KURZ, K., VON KÜGELGEN, I. & STARKE, K. (1993). Prejunctional modulation of noradrenaline release in mouse and rat vas deferens: contribution of P_1- and P_2-purinoceptors. *Br. J. Pharmacol.*, **110**, 1465–1472.

LIMBERGER, N., TRENDELENBURG, A.U. & STARKE, K. (1992). Pharmacological characterization of presynaptic α_2-autoreceptors in rat submaxillary gland and heart atrium. *Br. J. Pharmacol.*, **107**, 246–255.

LOHSE, M.J., KLOTZ, K.N., LINDENBORN-FOTINOS, J., REDDINGTON, M., SCHWABE, U. & OLSSON, R.A. (1987). 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) – a selective high affinity antagonist radioligand for A_1 adenosine receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **336**, 204–210.

MIYAHARA, H. & SUZUKI, H. (1987). Pre- and post-junctional effects of adenosine triphosphate on noradrenergic transmission in the rabbit ear artery. *J. Physiol.*, **389**, 423–440.

MOODY, C.J., MEGHJI, P. & BURNSTOCK, G. (1984). Stimulation of P_1-purinoceptors by ATP depends partly on its conversion to AMP and adenosine and partly on direct action. *Eur. J. Pharmacol.*, **97**, 47–54.

PADDLE, B.M. & BURNSTOCK, G. (1974). Release of ATP from perfused heart during coronary vasodilatation. *Blood Vessels*, **11**, 110–119.

RALEVIC, V. & BURNSTOCK, G. (1991). Roles of P_2-purinoceptors in the cardiovascular system. *Circulation*, **84**, 1–14.

RICHARDT, G., WAAS, W., KRANZHÖFER, R., MAYER, E. & SCHÖMIG, A. (1987). Adenosine inhibits exocytotic release of endogenous noradrenaline in rat heart: a protective mechanism in early myocardial ischemia. *Circ. Res.*, **61**, 117–123.

RUBINO, A., AMERINI, S., LEDDA, F. & MANTELLI, L. (1992). ATP modulates the efferent function of capsaicin-sensitive neurones in guinea-pig isolated atria. *Br. J. Pharmacol.*, **105**, 516–520.

SCHÜTZ, W., STRÖHER, M., FREISSMUTH, M., VALENTA, B. & SINGER, E.A. (1991). Adenosine receptors mediate a pertussis toxin-insensitive prejunctional inhibition of noradrenaline release on a papillary muscle model. *Naunyn-Schmied. Arch. Pharmacol.*, **343**, 311–316.

SEBASTIÃO, A.M., STONE, T.W. & RIBEIRO, J.A. (1990). The inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat: antagonism by 1,3,8-substituted xanthines. *Br. J. Pharmacol.*, **101**, 453–459.

SHINOZUKA, K., BJUR, R.A. & WESTFALL, D.P. (1988). Characterization of prejunctional purinoceptors on adrenergic nerves of the rat caudal artery. *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 221–227.

SPERLAGH, B. & VIZI, E.S. (1991). Effect of presynaptic P_2 receptor stimulation on transmitter release. *J. Neurochem.*, **56**, 1466–1470.

STARKE, K. (1987). Presynaptic α-autoreceptors. *Rev. Physiol. Biochem. Pharmacol.*, **107**, 73–146.

STARKE, K., GÖTHERT, M. & KILBINGER, H. (1989). Modulation of neurotransmitter release by presynaptic autoreceptors. *Physiol. Rev.*, **69**, 864–989.

STARKE, K., PESKAR, B.A., SCHUMACHER, K.A. & TAUBE, H.D. (1977). Bradykinin and postganglionic sympathetic transmission. *Naunyn-Schmied. Arch. Pharmacol.*, **299**, 23–32.

STOFFEL, D., VON KÜGELGEN, I. & STARKE, K. (1994). Presynaptic P_2-purinoceptors inhibiting the release of [^{3}H]-noradrenaline in rat heart atrium. *Naunyn-Schmied. Arch. Pharmacol.*, **350**, R19.

TODOROV, L.D., BJUR, R.A. & WESTFALL, D.P. (1994). Inhibitory and facilitatory effects of purines on transmitter release from sympathetic nerves. *J. Pharmacol. Exp. Ther.*, **268**, 985–989.

VON KÜGELGEN, I., KURZ, K., BÜLTMANN, R., DRIESSEN, B. & STARKE, K. (1994a). Presynaptic modulation of the release of the co-transmitters noradrenaline and ATP. *Fundam. Clin. Pharmacol.*, **8**, 207–213.

VON KÜGELGEN, I., KURZ, K. & STARKE, K. (1993). Axon terminal P_2-purinoceptors in feedback control of sympathetic transmitter release. *Neuroscience*, **56**, 263–267.

VON KÜGELGEN, I., KURZ, K. & STARKE, K. (1994b). P_2-purinoceptor-mediated autoinhibition of sympathetic transmitter release in mouse and rat vas deferens. *Naunyn-Schmied. Arch. Pharmacol.*, **349**, 125–134.

VON KÜGELGEN, I., SCHÖFFEL, E. & STARKE, K. (1989). Inhibition by nucleotides acting at presynaptic P_2-receptors of sympathetic neuro-effector transmission in the mouse isolated vas deferens. *Naunyn-Schmied. Arch. Pharmacol.*, **340**, 522–532.

VON KÜGELGEN, I., SPÄTH, L. & STARKE, K. (1992). Stable adenine nucleotides inhibit [^{3}H]-noradrenaline release in rabbit brain cortex slices by direct action at presynaptic adenosine A_1-receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **346**, 187–196.

VON KÜGELGEN, I., SPÄTH, L. & STARKE, K. (1994c). Evidence for P_2-purinoceptor-mediated inhibition of noradrenaline release in rat brain cortex. *Br. J. Pharmacol.*, **113**, 815–822.

VON KÜGELGEN, I. & STARKE, K. (1991). Noradrenaline-ATP co-transmission in the sympathetic nervous system. *Trends Pharmacol. Sci.*, **12**, 319–324.

WAKADE, A.R. & WAKADE, T.D. (1978). Inhibition of noradrenaline release by adenosine. *J. Physiol.*, **282**, 35–49.

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

WELFORD, L.A., CUSACK, N.J. & HOURNAI, S.M.O. (1986). ATP analogues and the guinea-pig taenia coli: a comparison of the structure-activity relationships of ectonucleotidases with those of the P_2-purinoceptor. *Eur. J. Pharmacol.*, **129**, 217–224.

WIKLUNG, N.P., GUSTAFSSON, L.E. & LUNDIN, J. (1985). Pre- and postjunctional modulation of cholinergic neuroeffector transmission by adenine nucleotides. Experiments with agonist and antagonist. *Acta Physiol. Scand.*, **125**, 681–691.

WILLIAMS, M., BRAUNWALDER, A. & ERICKSON, T.J. (1986). Evaluation of the binding of the A-1 selective adenosine radioligand, cyclopentyladenosine (CPA), to rat brain tissue. *Naunyn-Schmied. Arch. Pharmacol.*, **332**, 179–183.

ZIMMERMANN, H. (1994). Signalling via ATP in the nervous system. *Trends Neurosci.*, **17**, 420–426.

(Received December 1, 1994
Revised January 21, 1995
Accepted February 2, 1995)