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P_2-receptor modulation of noradrenergic neurotransmission in rat kidney

Christine Bohmann, *Ivar von Kügelgen & [1]L. Christian Rump

Medizinische Universitätsklinik Freiburg, Innere Medizin IV, Hugstetter Strasse 55, D-79106 Freiburg, and *Pharmakologisches Institut, Hermann-Herder-Strasse 5, D-79104 Freiburg, Germany

1 ATP has previously been shown to act as a sympathetic cotransmitter in the rat kidney. The present study analyses the question of whether postganglionic sympathetic nerve endings in the kidney possess P_2-receptors which modulate noradrenaline release. Rat kidneys were perfused with Krebs-Henseleit solution containing the noradrenaline uptake blockers cocaine and corticosterone and the α_2-adrenoceptor antagonist rauwolscine. The renal nerves were electrically stimulated, in most experiments by 30 pulses applied at 1 Hz. The outflow of endogenous noradrenaline (or, in some experiments, of ATP and lactate dehydrogenase) as well as the perfusion pressure were measured simultaneously.

2 The P_2-receptor agonist adenosine-5′-O-(3-thiotriphosphate) (ATPγS, 3–30 μM) reduced the renal nerve stimulation (RNS)-induced outflow of noradrenaline (estimated $EC_{50} = 8$ μM). The P_2-receptor antagonist cibacron blue 3GA (30 μM) shifted the concentration-inhibition curve for ATPγS to the right (apparent pK_B value 4.7).

3 Cibacron blue 3GA (3–30 μM) and its isomer reactive blue 2 (3–30 μM) significantly increased RNS-induced outflow of noradrenaline in the presence of the P_1-receptor antagonist 8-(*p*-sulphophenyl)theophylline (8-SPT, 100 μM) by about 70% and 90%, respectively. The P_2-receptor antagonist suramin (30–300 μM) only tended to enhance RNS-induced outflow of noradrenaline. When the nerves were stimulated by short pulse trains consisting of 6 pulses applied at 100 Hz (conditions under which autoinhibition is inoperative), reactive blue 2 did not affect the RNS-induced outflow of noradrenaline.

4 RNS (120 pulses applied at 4 Hz) induced the outflow of ATP but not of the cytoplasmatic enzyme lactate dehydrogenase.

5 ATPγS (3–30 μM) concentration-dependently reduced pressor responses to RNS at 1 Hz. Cibacron blue 3GA, reactive blue 2 as well as suramin also reduced pressor responses to RNS (maximally by 50 to 70%).

6 This study in rat isolated kidney, in which the release of endogenous noradrenaline was measured, demonstrates that renal sympathetic nerves possess prejunctional P_2-receptors that mediate inhibition of transmitter release. These prejunctional P_2-receptors are activated by endogenous ligands, most likely ATP, released upon nerve activity. Both, P_2-receptor agonists and P_2-receptor antagonists reduced pressor responses to RNS either by inhibiting transmitter release or by blocking postjunctional vasoconstrictor P_2-receptors.

Keywords: P_2-receptors; autoreceptors; noradrenaline release; ATP release; lactate dehydrogenase release; rat kidney

Introduction

Renal postganglionic sympathetic nerves use adenosine 5′-triphosphate (ATP) and noradrenaline as cotransmitters (Schwartz & Malik, 1989; Rump *et al*., 1990; 1992; Bohmann *et al*., 1995; for reviews see Burnstock, 1990; von Kügelgen & Starke, 1991; Zimmermann, 1994). Neuronally released ATP activates postjunctional vasoconstriction-mediating P_{2X}-receptors (Schwartz & Malik 1989; Rump *et al*., 1990; 1992; Bohmann *et al*., 1995) and possibly also vasodilatation-mediating P_{2Y}-receptors (Churchill & Ellis 1993; Inscho *et al*., 1994; Bohmann *et al*., 1996a), which have been demonstrated in renal tissues of various species.

The sympathetic axons in the kidney are endowed with a variety of prejunctional receptors which when activated enhance or inhibit transmitter release (Starke, 1987; Rump, 1987; Rump & Schollmeyer, 1989; Starke *et al*., 1989; Bohmann *et al*., 1993a,b). These prejunctional receptors include α_2-adrenoceptors activated by noradrenaline (see Starke, 1987; Starke *et al*., 1989) and P_1-receptors of the A_1-subtype activated by adenosine (see Fredholm & Dunwiddie, 1988); both of these receptors mediate inhibition of transmitter release. Recently, noradrenergic axons have been shown to possess prejunctional P_2-receptors for ATP. In tissues like the mouse vas deferens and the rat iris, heart atrium, tail artery and brain cortex activation of P_{2Y}-like P_2-receptors by exogenous agonists decreases the release of noradrenaline (von Kügelgen *et al*., 1989; 1994a,b; 1995; Fuder & Muth, 1993; Kurz *et al*., 1993; Gonçalves & Queiroz, 1996; see von Kügelgen, 1996). Even more importantly, in the sympathetically innervated tissues mentioned, P_2-receptor antagonists increase the release of noradrenaline, indicating that the prejunctional P_2-receptors are activated by endogenous ligands and modulate sympathetic transmitter release by a negative feedback mechanism (Fuder & Muth, 1993; von Kügelgen *et al*., 1993; 1994a; 1995). In contrast to the inhibitory action of adenine nucleotides, in some preparations activation of P_2-receptors has been shown to enhance transmitter release (Miyahara & Suzuki, 1987; Sperlagh & Vizi, 1991; Allgaier *et al*., 1995).

The present study was designed to investigate whether prejunctional P_2-receptors occur at the sympathetic axons in the kidney and play a physiological role in modulation of noradrenaline release from renal nerves. For this purpose, effects of purinoceptor ligands on the release of endogenous noradrenaline from the rat isolated kidney were examined. In additional experiments, the outflow of ATP and of the cytoplasmatic enzyme lactate dehydrogenase from perfused kidneys was determined. Some of the results have been presented to the German Pharmacological Society (Bohmann *et al*., 1996b).

[1] Author for correspondence.

Methods

Male Wistar rats (Charles River, Sulzfeld, Germany) 14–16 weeks of age (weighing 374±14 g, $n=53$) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}) intraperitoneally. Kidneys were isolated and perfused with Krebs-Henseleit solution as described previously (Rump *et al*., 1992). Bipolar platinum electrodes were placed around the renal arteries to stimulate the renal sympathetic nerves. Perfusion pressure was monitored continuously with a Statham P23 DB pressure transducer (Gould Inc) coupled to a Watanabe pen recorder (Graphtec Corp).

Outflow of endogenous noradrenaline

Kidneys were perfused with warmed (37°C) and oxygenated (95% O_2, 5% CO_2) Krebs-Henseleit solution containing corticosterone (20 μM) at a constant rate of 6 ml min^{-1} for 60 min. After this stabilization period a control stimulation with 150 pulses at 5 Hz (1 ms pulse width, 50 V) was applied and cocaine (10 μM) as well as rauwolscine (0.1 μM) were added to the perfusion solution. After 25 min 1-min fractions of the perfusate were collected into vials containing 167 μl of 1 M HCl, 13.3 μl of 0.067 M EDTA and 3.3 μl of 1 M NaS_2O_3. Four electrical stimulation periods (S_1 through to S_4) with either 30 pulses at 1 Hz or 6 pulses at 100 Hz (1 ms pulse width, 50 V) were applied 2, 23, 44 and 65 min after the start of fraction collection. Drugs were added to the perfusion solution either at increasing concentrations 16 min before S_2 to S_4, or 25 min before the start of fraction collection, or (indomethacin) throughout perfusion.

The noradrenaline content in the samples was determined, after adsorption onto alumina and elution by $HClO_4$ by high performance liquid chromatography with a reversed phase column and electrochemical detection (h.p.l.c.-e.c.d.) (Bohmann *et al*., 1995). The amount of noradrenaline in each sample was calculated from a standard calibration curve (30 to 3000 pg noradrenaline), corrected for recoveries and expressed as pmol g^{-1} kidney. Recovery was 62±0.6% ($n=140$).

Calculations

Renal nerve stimulation (RNS)-induced outflow of noradrenaline was determined by subtracting the estimated spontaneous outflow of noradrenaline from the noradrenaline content in the three 1-min samples collected immediately after onset of stimulation. The spontaneous outflow of noradrenaline was taken as the noradrenaline content present in the 1-min sample collected before onset of stimulation. S_1 served as a reference stimulation and the RNS-induced outflow of noradrenaline in S_2-S_4 is expressed as a ratio of that in S_1 (S_n/S_1). Pressor responses to RNS or to the P_2-receptor agonist ATPγS were measured as the maximum increase in perfusion pressure above basal perfusion pressure (mm Hg). Pressor responses induced by S_2 to S_4 are expressed as a ratio of that induced by S_1 (S_n/S_1).

For further evaluation of drug effects on RNS-induced outflow of noradrenaline and pressor responses to RNS, the respective S_n/S_1 ratios were calculated as a percentage of the values determined in the corresponding control experiments.

The EC_{50} value of ATPγS is the concentration that decreased RNS-induced outflow of noradrenaline by 50%. The apparent pK_B ($-\log K_B$) value of cibacron blue 3GA against ATPγS was calculated from the increase in EC_{50} values by use of equation 4 of Furchgott (1972).

Outflow of endogenous ATP and lactate dehydrogenase

The kidneys were perfused with drug-free Krebs-Henseleit solution as described above. After a stabilization period of 60 min seven 1-min fractions of the perfusate were collected and stored on ice. There was one stimulation period (S_1) with 120 pulses at 4 Hz (1 ms pulse width, 50 V).

The ATP content in the samples was determined by use of the ATP bioluminescence FL-AAM assay kit (Sigma, Deisenhofen, Germany) and a Biolumat LB 953 luminometer (Berthold, Wildbad, Germany) (see Bohmann *et al*., 1995). The lactate dehydrogenase (LDH) activity in the samples was determined by use of the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Mannheim, Germany). Absorbance was read at 490 nm.

Calculations

The amount of ATP present in each sample was determined by reference to a standard calibration curve (1 pM–5 nM, obtained with Krebs-Henseleit solution as solvent), corrected for recoveries (average recovery of ATP was 19±8%, $n=8$, see Bohmann *et al*., 1995) and expressed as pmol g^{-1} kidney. The LDH activity in each sample was expressed as a percentage of the maximal LDH efflux following addition of 1% Triton X-100 (Sigma) to the perfusion solution. Determination of a maximal ATP efflux from the kidneys following treatment with Triton X-100 was not possible due to an interaction of Triton X-100 with either the ATP assay or extracellular ATP breakdown.

Materials

The Krebs-Henseleit solution had the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, KH_2PO_4 1.03, D-(+)-glucose 11.1, Na_2 EDTA 0.067 and ascorbic acid 0.07. The following drugs were purchased: (±)-noradrenaline HCl, adenosine-5′-*O*-(3-thiotriphosphate) (ATPγS), cibacron blue 3GA, corticosterone and indomethacin (Sigma), cocaine HCl (Merck, Darmstadt, Germany), rauwolscine HCl (Roth, Karlsruhe, Germany), 8-(*p*-sulphophenyl)theophylline (8-SPT), pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium salt (PPADS) and reactive blue 2 (Biotrend, Research Biochemicals, Köln, Germany). The following drug was kindly donated: suramin hexasodium salt (Bayer, Wuppertal, Germany). Drugs were dissolved either in distilled water or absolute ethanol (corticosterone and indomethacin) before being diluted with Krebs-Henseleit solution.

Statistics

Means±s.e.mean are presented throughout. Differences between means were tested for significance by unpaired Student's *t* test. Probability levels of less than 0.05 were considered statistically significant. For multiple comparisons with the same control, *P* levels were adjusted according to Bonferroni; *n* is the number of kidneys. A significant effect of treatment with P_1- and P_2-receptor antagonists or indomethacin was tested by two-way ANOVA.

Results

RNS-induced outflow of noradrenaline and pressor responses to RNS

Rat kidneys were isolated and perfused with Krebs-Henseleit solution. Renal sympathetic nerves were electrically stimulated four times and the RNS-induced outflow of endogenous noradrenaline as well as pressor responses to RNS were measured. Unless stated otherwise each stimulation period (S_1 to S_4) consisted of 30 pulses applied at 1 Hz. In the presence of corticosterone (20 μM), cocaine (10 μM) and rauwolscine (0.1 μM) the outflow of endogenous noradrenaline evoked by the first period of stimulation (S_1) was 15.5±1.1 pmol g^{-1} kidney and the pressor response evoked by S_1 amounted to 143.9±6.7 mmHg ($n=39$; Table 1). When solvent was added before S_2 to S_4 the spontaneous and the RNS-induced outflow

Table 1 Outflow of endogenous noradrenaline (NA) and pressor responses induced by renal nerve stimulation (S_1)

	NA (pmol g^{-1} kidney)	*Pressor Responses* (mmHg)	n
No drug	15.5±1.1	143.9±6.7	39
Cibacron blue 3GA (30 μM)	29.4±2.8*	37.5±8.6*	8
8-SPT (100 μM)	16.3±1.6	140.0±8.6	17
8-SPT (100 μM)+ indomethacin (10 μM)	12.7±1.4	88.1±11.3**	8
8-SPT (100 μM) (6 pulses at 100 Hz)	1.4±0.2***	37.5±6.1***	10

Rat renal sympathetic nerves were stimulated by 30 pulses at 1 Hz or, when indicated, by 6 pulses at 100 Hz (1 ms pulse width, 50 V). Drugs were added either 25 min before the start of fraction collection or (indomethacin) throughout perfusion (8-SPT, 8-(*p*-sulphophenyl)theophylline). *Significant difference compared with no drug experiments, $P<0.05$; **significant difference compared with 8-SPT experiments, $P<0.05$; ***Significant difference compared with stimulation by 30 pulses at 1 Hz, $P<0.05$; modified *t*-test according to Bonferroni.

of noradrenaline (Figure 1a) as well as the evoked pressor responses (not shown) remained constant.

Effects of ATPγS and its interaction with cibacron blue 3GA

The P_2-receptor agonist ATPγS added at increasing concentrations (3 to 30 μM) before S_2 to S_4 did not change the spontaneous outflow of noradrenaline but concentration-dependently inhibited the RNS-induced outflow (Figures 1b and 2). From the concentration-inhibition curve an EC_{50} value (concentration causing inhibition by 50%) of 8 μM was estimated for ATPγS. The P_2-receptor antagonist cibacron blue 3GA (30 μM; Shirahase *et al*., 1991; Boland *et al*., 1992) added from 25 min before the start of collection did not change the spontaneous outflow of noradrenaline (not shown) but markedly increased the RNS-induced outflow (Table 1). Cibacron blue 3G shifted the concentration-inhibition curve of ATPγS to the right (Figure 2). From the shift to the right an apparent pK_B value of 4.7 was estimated for cibacron blue 3GA (see Methods).

In addition to changing the RNS-induced outflow of noradrenaline ATPγS elicited fast and transient increases in the perfusion pressure (maximal peak increase 155±12.8 mmHg; $n=6$), which returned to baseline before the next stimulations (S_2 to S_4) were applied (not shown in Figure 1c due to time scale of the plot). Moreover, ATPγS (3 to 30 μM) decreased the pressor responses to RNS (Figure 1c; Table 2). Cibacron blue 3GA added 25 min before the start of collection decreased the pressor response to S_1 (Table 1) and inhibited the increases in perfusion pressure induced by ATPγS by about 30% (not shown). Since the RNS-induced pressor responses were already markedly decreased in the presence of cibacron blue 3GA, the effects of ATPγS on these responses could not be determined.

Effects of P_1- and P_2-receptor antagonists

The effects of P_1- and P_2-receptor antagonists on the responses to RNS were also studied in experiments in which the antagonists were added after S_1 so that the responses to S_1 served as reference values. In these experiments the P_1-receptor antagonist 8-(*p*-sulphophenyl)theophylline (8-SPT; 10 to 100 μM; Fredholm *et al*., 1994) only slightly enhanced the RNS-induced outflow of noradrenaline by maximally 25±11% ($n=5$; Figure 3a). The P_2-receptor antagonist suramin (30 to 300 μM; Hoyle *et al*., 1990; Kennedy, 1990; Fredholm *et al*., 1994) at best tended to increase RNS-induced outflow of noradrenaline (by 11±6%; $n=7$; not shown). In contrast, reactive blue 2 (3 to 30 μM) an isomer of cibacron blue 3GA which also blocks P_{2Y}-receptors preferentially in some tissues (Burnstock & Warland, 1987; Kennedy, 1990) markedly enhanced RNS-induced outflow of noradrenaline, by maximally 140% (Figures 3b and 4a). 8-SPT, suramin and reactive blue 2 did not change the spontaneous outflow of noradrenaline (not shown), but PPADS (10 to 100 μM), a preferential P_{2X}-receptor antagonist (Lambrecht *et al*., 1992; Mc Laren *et al*., 1993), caused about a 20 fold increase in the spontaneous outflow of noradrenaline. Therefore, the effects of PPADS on RNS-induced outflow of noradrenaline could not be determined.

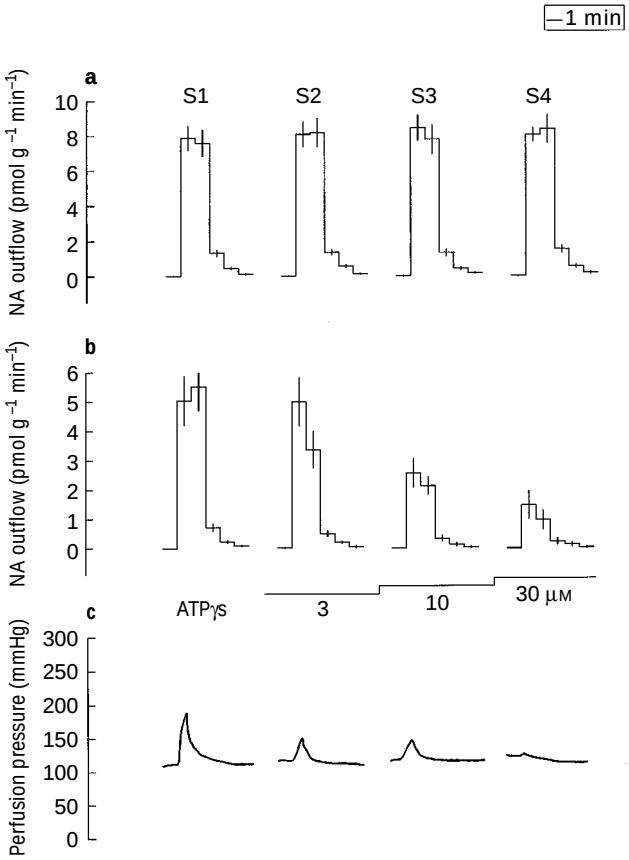

Figure 1 Plots show the effect of ATPγS on RNS-induced outflow of noradrenaline (NA) and on pressor responses in rat kidneys. There were four stimulation periods (S_1 to S_4; each with 30 pulses at 1 Hz; 1 ms pulse width, 50 V). (a) NA outflow g^{-1} kidney and min^{-1} as mean±s.e.mean for control experiments ($n=12$). (b) NA outflow g^{-1} kidney and min^{-1} as mean±s.e.mean for experiments with ATPγS ($n=6$). ATPγS was added at the concentrations indicated 16 min before S_2 to S_4. (c) Representative perfusion pressure trace from one experiment with ATPγS. (The initial transient pressor responses to ATPγS are not shown due to the time scale of the plot.)

The effects of reactive blue 2 and its isomer cibacron blue 3GA were further studied in the presence of 8-SPT (100 μM) or a combination of 8-SPT and indomethacin (10 μM; see Rump *et al*., 1990) in order to avoid any interaction with P_1-receptors or endogenous prostaglandins. Neither 8-SPT nor indomethacin caused any significant change in the outflow of noradrenaline induced by S_1 (Table 1). In the presence of 8-SPT (100 μM) reactive blue 2 and cibacron blue 3GA (each 3 to

30 μM) enhanced the RNS-induced outflow of noradrenaline maximally by 93 and 71%, respectively (Figure 4a, b). Hence, the reactive blue 2-induced increase in the evoked outflow of noradrenaline was lower in the presence of 8-SPT than in its absence (Figure 4a). In the presence of the combination of 8-SPT (100 μM) and indomethacin (10 μM) reactive blue 2 (3 to 30 μM) enhanced the RNS-induced outflow of noradrenaline to a very similar extent when compared to experiments in the presence of 8-SPT alone. The maximal increase by reactive blue 2 (30 μM) in the combined presence of 8-SPT and indomethacin amounted to $98 \pm 38\%$ ($n=4$).

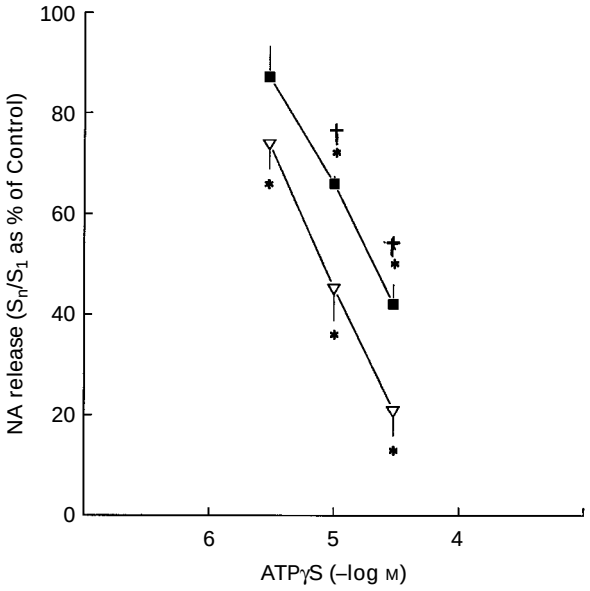

Figure 2 Line graphs showing effect of ATPγS on RNS-induced noradrenaline (NA) release in rat kidneys and its interaction with the P_2-receptor antagonist cibacron blue 3GA (30 μM). There were four stimulation periods (S_1 to S_4; each with 30 pulses at 1 Hz; 1 ms pulse width, 50 V). ATPγS was added at increasing concentrations 16 min before S_2 to S_4. Cibacron blue 3GA (30 μM) was added 25 min before the start of fraction collection. Results are expressed as ratios S_n/S_1 and are given as percentages of the ratios of the corresponding controls. ATPγS alone ($\triangledown$) and in the presence of cibacron blue 3GA (■). Means are shown for 4 to 6 experiments; vertical lines indicate s.e.mean. *Significant difference compared with control, $P<0.05$; modified t test according to Bonferroni. †Significant effect of treatment with cibacron blue 3GA, $P<0.05$; ANOVA.

In some additional experiments the nerves were stimulated not by 30 pulses at 1 Hz but by very short pulse trains consisting of 6 pulses applied at 100 Hz. Under these stimulation conditions autoinhibition of transmitter release in rat isolated kidney does not operate (Bohmann *et al*., 1993a). When the nerves were stimulated by 6 pulses at 100 Hz the responses to S_1 were markedly lower but still detectable (Table 1). In these experiments reactive blue 2 (3 to 30 μM) caused no significant increase in RNS-induced outflow of noradrenaline (Figure 4a).

The P_2-receptor antagonists reactive blue 2, cibacron blue 3GA and suramin decreased pressor responses to RNS when they were added after S_1; PPADS had no effects (Table 2).

RNS-induced outflow of ATP and lactate dehydrogenase

In a final series of experiments the possible effect of electrical stimulation on outflow of ATP and LDH from perfused kidneys was studied. There was a basal efflux of ATP as well as of LDH. However, RNS by 120 pulses at 4 Hz increased only the

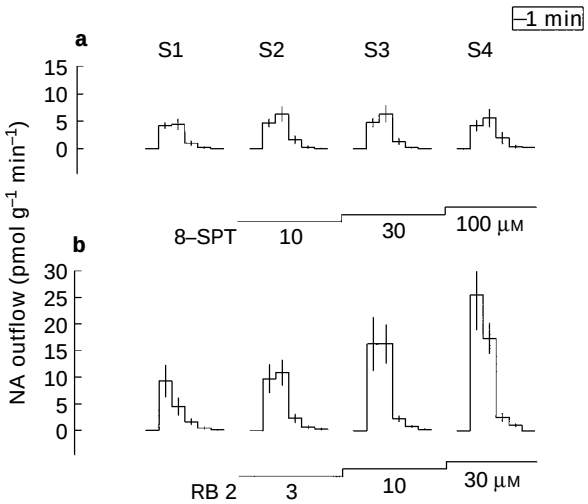

Figure 3 Plots show the effects of the P_1-receptor antagonist 8-(*p*-sulphophenyl)theophylline (8-SPT) and the P_2-receptor antagonist reactive blue 2 (RB 2) on RNS-induced outflow of noradrenaline (NA) from rat kidneys. There were four stimulation periods (S_1 to S_4; each with 30 pulses at 1 Hz; 1 ms pulse width, 50 V). 8-SPT (a) ($n=6$) or RB 2 (b) ($n=5$) was added at increasing concentrations 16 min before S_2 to S_4. Plots are NA outflow g^{-1} kidney and min^{-1} given as mean$\pm$s.e.mean.

Table 2 Effects of P_1- and P_2-receptor ligands on evoked pressor responses

	S_2/S_1 (%)	S_3/S_1 (%)	S_4/S_1 (%)	n
Agonist				
ATPγS (3, 10, 30 μM)	43.4$\pm$5.2*	42.7$\pm$11.5*	9.6$\pm$4.4*	6
Antagonists				
Suramin (30, 100, 300 μM)	70.0$\pm$7.0*	61.3$\pm$7.1*	47.4$\pm$8.0	7
PPADS (10, 30, 100 μM)	99.2$\pm$1.8	98.1$\pm$1.6	95.2$\pm$2.3	3
8-SPT (10, 30, 100 μM)	106.6$\pm$4.0	109.7$\pm$5.1	112.3$\pm$7.1	5
Reactive blue (3, 10, 30 μM)	88.6$\pm$3.3*	74.3$\pm$7.3*	46.2$\pm$10.4*	5
Antagonists in the presence of 8-SPT (100 μM)				
Reactive blue 2 (3, 10, 30 μM)	83.5$\pm$5.7*	64.0$\pm$6.0*	32.9$\pm$3.4*	5
Cibacron blue 3GA (3, 10, 30 μM)	68.0$\pm$5.8*	47.5$\pm$5.0*	28.0$\pm$3.2*	5
Reactive blue 2 (3, 10, 30 μM) (6 pulses at 100 Hz)	93.4$\pm$3.4	68.5$\pm$7.1*	21.5$\pm$3.2*	4

Rat renal sympathetic nerves were stimulated four times by 30 pulses at 1 Hz or, when indicated, by 6 pulses at 100 Hz (1 ms pulse width, 50 V). Results are expressed as ratios S_n/S_1 and are given as percentages of the ratios of the corresponding controls. Drugs were added either at increasing concentrations 16 min before S_2 to S_4 or (8-SPT only) 25 min before the start of fraction collection (8-SPT, 8-(*p*-sulphophenyl)theophylline; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid). *Significant difference compared with respective controls, $P<0.05$; modified t test according to Bonferroni.

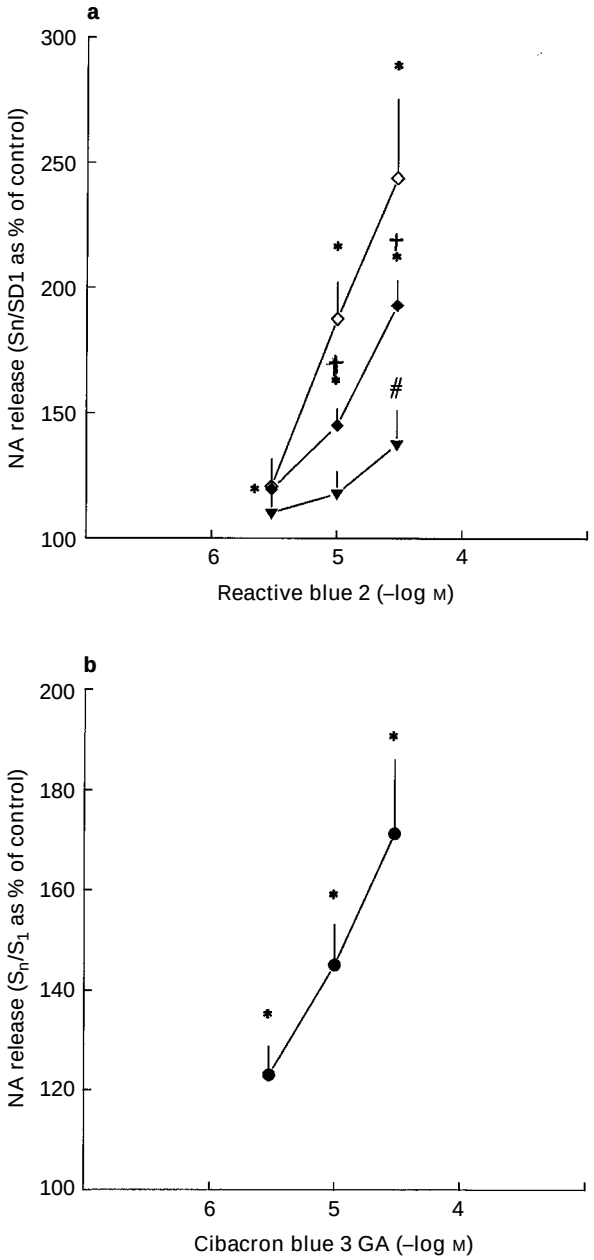

Figure 4 Line graphs show the effects of reactive blue 2 (a) and cibacron blue 3GA (b) on RNS-induced noradrenaline (NA) release in rat kidneys in either the absence or presence of 8-(*p*-sulphophenyl)theophylline (8-SPT, 100 μM). There were four stimulation periods (S_1 to S_4; each either with 30 pulses at 1 Hz or with 6 pulses at 100 Hz; 1 ms pulse width, 50 V). Reactive blue 2 (RB 2) and cibacron blue 3GA were added at increasing concentrations 16 min before S_2 to S_4. 8-SPT was added 25 min before the start of fraction collection. Results are expressed as ratios S_n/S_1 and are given as percentages of the ratios of the corresponding controls. (a) RB 2 alone (30 pulses at 1 Hz; $n=5$, ◇), RB 2 in the presence of 8-SPT (30 pulses at 1 Hz; $n=6$, ◆; 6 pulses at 100 Hz; $n=5$, ▼). (b) Cibacron blue 3GA in the presence of 8-SPT (30 pulses at 1 Hz; $n=4$). Means are shown and vertical lines indicate s.e.mean. *Significant difference compared with controls, $P<0.05$; modified *t* test according to Bonferroni. †Significant effect of treatment with 8-SPT, $P<0.05$; ANOVA. #Significant difference between different stimulation frequencies, $P<0.05$; *t* test.

efflux of ATP but not that of LDH (Figure 5). (Since it has previously been shown that the RNS-induced ATP outflow was frequency-dependent in rat kidney (Bohmann *et al.*, 1995), a stimulation frequency of 4 Hz instead of 1 Hz was chosen for these experiments).

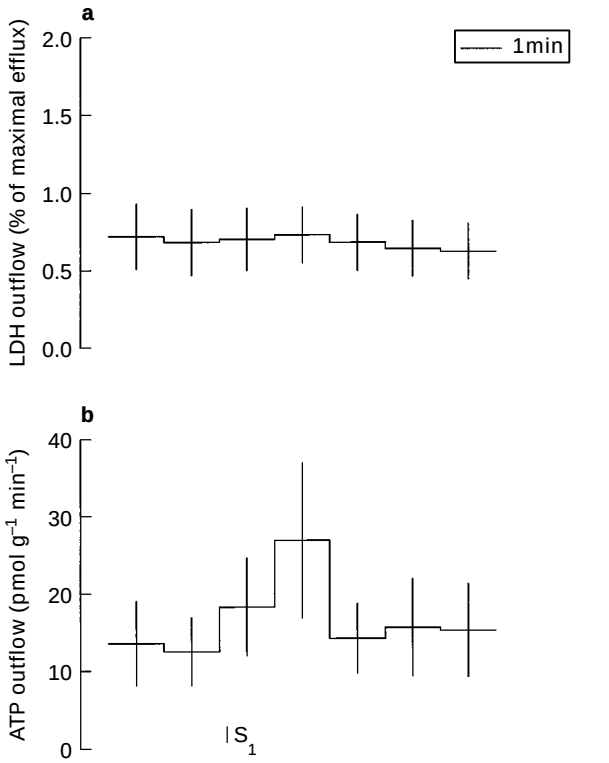

Figure 5 Plots show the outflow of lactate dehydrogenase (LDH) and ATP and the effect of nerve stimulation. There was one stimulation period (S_1; with 120 pulses at 4 Hz; 1 ms pulse width, 50 V). (a) LDH outflow min^{-1} as percentage of maximal efflux induced by 1% Triton X-100. (b) ATP outflow g^{-1} kidney and min^{-1}. Means ± s.e.mean are shown from 8 experiments.

Discussion

ATP and noradrenaline are sympathetic cotransmitters in the rat kidney (see Introduction). This has been shown not only by measuring effector responses but also by the biochemical analysis of transmitter release. Nerve stimulation induced the outflow of both endogenous noradrenaline and endogenous ATP from perfused rat kidneys (Bohmann *et al.*, 1995 and present study) but not that of the cytoplasmatic enzyme LDH (present study). This indicates that the outflow of ATP and noradrenaline due to nerve activity is a physiological event and not due to cellular destruction in this *in vitro* system.

The aim of the present study was to investigate whether prejunctional P_2-receptors occur at sympathetic axons in the kidney and whether they play a physiological role in modulating transmitter release. For this purpose effects of P_2-receptor ligands on the outflow of endogenous noradrenaline evoked by short or long trains of electrical pulses were analysed. To exclude an interaction of P_2-receptor antagonists and agonists with cellular noradrenaline uptake mechanisms and α_2-adrenoceptors, the experiments were performed in the presence of cocaine, corticosterone and rauwolscine, respectively. A P_2-receptor-mediated modulation of the evoked ATP outflow could not be studied due to the known interference of P_2-receptor ligands with either the determination or the metabolism of ATP (see von Kügelgen, 1996).

Prejunctional P_2-receptors and noradrenaline release

In a previous study in the rat isolated kidney it has been shown that RNS at 1 Hz, which is in the average physiological range, induces the release of substantial amounts of endogenous noradrenaline (Bohmann *et al.*, 1994). In the present study, with the same experimental conditions, the metabolically

stable ATP analogue and P_2-receptor agonist ATPγS (Welford *et al*., 1986) concentration-dependently inhibited RNS-induced noradrenaline release. The P_2-receptor antagonist cibacron blue 3GA markedly shifted the concentration-response curve for ATPγS to the right, suggesting that the inhibitory effect of ATPγS was in fact due to activation of prejunctional P_2-receptors. The estimated pK_B value for cibacron blue 3GA at prejunctional P_2-receptors in the rat kidney was 4.7 which is similar to pK_B values obtained for cibacron blue 3GA at prejunctional P_2-receptors in other tissues (e.g., rat brain cortex 5.0, von Kügelgen *et al*., 1994b; rat atria 5.1, von Kügelgen *et al*., 1995). Taken together, these results strongly suggest that rat renal sympathetic postganglionic nerve terminals are endowed with inhibitory P_2-receptors which resemble the P_{2Y}-like P_2-receptors found at noradrenergic axons in other tissues of the rat and the mouse (see von Kügelgen, 1996).

Physiological role of prejunctional P_2-receptors

In accord with previous results in the rat isolated kidney (Bohmann *et al*., 1993b), the P_1-receptor antagonist 8-SPT when given after the reference stimulation period S_1 slightly increased the RNS-induced outflow of noradrenaline. Thus, prejunctional P_1-receptors, probably of the A_1-subtype, seem to be activated by endogenous adenosine. Blockade of these receptors increases transmitter release.

The present study clearly demonstrates that the prejunctional P_2-receptors play an even more pronounced physiological role in rat renal neurotransmission. Both P_2-receptor antagonists, reactive blue 2 and its isomer cibacron blue 3GA, markedly enhanced the RNS-induced outflow of endogenous noradrenaline. Since in the present study the noradrenaline uptake mechanisms, as well as α_2-adrenoceptors, were blocked and an increase was observed in the absence and presence of blockade of P_1-receptors by 8-SPT these results indicate that the prejunctional P_2-receptors were activated by endogenous ligands, most likely by endogenous ATP. This view was further supported by the finding that reactive blue 2 enhanced the RNS-induced outflow of noradrenaline to a very similar extent even after blockade of prostaglandin synthesis by indomethacin.

The physiological function of prejunctional inhibitory P_2-receptors was further tested. Autoinhibition via prejunctional receptors operates with a latency due to the fact that transmitter substances released have to activate prejunctional receptors before inhibition of subsequent transmitter release (Story *et al*., 1981; Singer, 1988; Starke *et al*., 1989). Accordingly, it has previously been shown in the rat kidney that upon stimulation with very short pulse trains of 6 pulses at 100 Hz the release of noradrenaline is not influenced by α_2-autoinhibition (Bohmann *et al*., 1993a). With the same stimulation protocol, the P_2-receptor antagonist reactive blue 2 failed to cause a significant increase in RNS-induced noradrenaline release, indicating that the endogenous adenine nucleotides activating the prejunctional P_2-receptors are released upon nerve activity. Similar conditions of operation have previously been shown for the prejunctional P_2-receptors in the mouse vas deferens and the rat atrium indicating a function of these P_2-receptors as autoreceptors (von Kügelgen *et al*., 1993; 1995). The failure of reactive blue 2 to enhance transmitter release under autoinhibition free conditions further indicates that the increase by reactive blue 2 is due to an interaction with P_2-receptors and not due to unspecific effects.

The perfusate from isolated and perfused kidneys contains relatively high amounts of ATP (Bohmann *et al*., 1995 and present results) indicating that there is a spontaneous efflux of ATP in the kidney. However, our data demonstrated that the prejunctional P_2-receptors are at least predominantly activated by ATP released upon nerve activity and not by ATP continuously present in the biophase. In the rat kidney a marked part of the ATP released by nerve stimulation has been shown to be derived from non-neuronal cells due to the transmitter action of released noradrenaline on α_1-adrenoceptors (Bohmann *et al*., 1995). Hence, prejunctional P_2-receptors could be activated by both cotransmitter ATP as well as non-neuronally derived ATP. Notwithstanding, the prejunctional P_2-receptors in the rat kidney will mediate a negative feedback of sympathetic transmitter release and operate as a kind of autoreceptor.

8-SPT added throughout the experiment attenuated the facilitatory effect of reactive blue 2. The reason for this attenuation is not known but might be due to an interaction between prejunctional P_1- and P_2-receptors. Previously, evidence for the occurrence of a common receptor for adenine nucleosides and nucleotides which is blocked by 8-SPT as well as by α,β-methylene-ATP has been obtained (Shinozuka *et al*., 1988; 1990; King *et al*., 1996). Even though an involvement of such a receptor cannot be ruled out, the marked increase in the RNS-induced outflow of noradrenaline by reactive blue 2 and cibacron blue 3GA in the presence of 8-SPT indicates that endogenous adenine nucleotides acted at least predominantly via a receptor selective for nucleotides, a P_2-receptor.

In the present experiments in the rat kidney suramin only tended to enhance the RNS-induced outflow of noradrenaline. A likely reason is that suramin has a low affinity for prejunctional P_2-receptors in rat tissues as previously suggested for the prejunctional P_2-receptor in rat iris (Fuder & Muth, 1993), rat brain cortex (von Kügelgen *et al*., 1994b), rat vas deferens (Kurz *et al*., 1993; von Kügelgen *et al*., 1994a), rat heart atrium (von Kügelgen *et al*., 1995) and rat tail artery (Goncalves & Queiroz, 1996). The effect of the preferential P_{2X}-receptor antagonist PPADS on RNS-induced outflow of noradrenaline could not be determined, since PPADS markedly enhanced basal outflow of endogenous noradrenaline, probably due to effects unrelated to an action on P_2-receptors (Connolly, 1995).

Pressor responses to RNS

Previous studies in the rat isolated kidney have shown that pressor responses to RNS are not exclusively mediated by noradrenaline, but also by ATP; the purinergic component is most prominent at low renal nerve activity (Schwartz & Malik, 1989; Rump *et al*., 1990; 1992; Bohmann *et al*., 1995). Accordingly, the P_2-receptor antagonists reactive blue 2, cibacron blue 3GA and suramin concentration-dependently decreased pressor responses to RNS in the present study. Moreover, ATPγS concentration-dependently enhanced basal perfusion confirming the occurence of vasoconstrictor P_2-receptors in rat kidney (Schwartz & Malik, 1989; Rump *et al*., 1990; 1992; Churchill & Ellis, 1993; Bohmann *et al*., 1995). The inhibition of RNS-induced pressor responses by ATPγS, on the other hand, seems to be mainly due to the inhibition of RNS-induced outflow of noradrenaline.

ATPγS and the P_2-receptor antagonists reactive blue 2, cibacron blue 3GA and suramin all inhibited RNS-induced pressor responses but affected the RNS-induced outflow of noradrenaline in a different manner, indicating that both responses are not directly related. In other words, changes in the outflow of noradrenaline are unlikely to be due to a change in the perfusion of the kidneys.

Conclusion

Postganglionic sympathetic nerve endings of the rat kidney possess release-inhibiting P_2-receptors, presumably belonging to the group of G-protein coupled P_{2Y}-receptors. These P_2-receptors are activated by endogenous adenine nucleotides and seem to operate as a kind of autoreceptor. This view is supported by (1) the interruption of the negative inhibitory feedback by P_2-receptor antagonists and (2) the dependence of this modulatory mechanism on the stimulation conditions.

Thus, rat renal sympathetic nerve endings possess P_2-receptors operating as a kind of autoreceptor in addition to α_{2D}-autoreceptors (Bohmann *et al*., 1994) to inhibit noradrenaline release. Activation of prejunctional α_2-adrenoceptors has been shown to reduce ATP release in the rat kidney as well as in

other tissues (Bohmann *et al*., 1995; see von Kügelgen, 1996). It is still an open question whether activation of prejunctional P_2-receptors also modulates the release of ATP from sympathetic axons (see von Kügelgen, 1996).

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