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Evidence for P_2-purinoceptor-mediated inhibition of noradrenaline release in rat brain cortex

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1 Some postganglionic sympathetic axons possess P_{2Y}-like P_2-purinoceptors which, when activated, decrease the release of noradrenaline. We examined the question of whether such receptors also occur at the noradrenergic axons in the rat brain cortex. Slices of the brain cortex were preincubated with [^{3}H]-noradrenaline, then superfused with medium containing desipramine (1 μM) and stimulated electrically, in most experiments by trains of 4 pulses/100 Hz.

2 The selective adenosine A_1-receptor agonist, N^6-cyclopentyl-adenosine (CPA; 0.03–3 μM) as well as the non-subtype-selective agonist 5'-N-ethylcarboxamido-adenosine (NECA; 0.3–3 μM) reduced the evoked overflow of tritium, whereas the adenosine A_{2a}-receptor agonist, 2-*p*-(2-carbonylethyl)-phenethylamino-5'-N-ethylcarboxamido-adenosine (CGS-21680; 0.003–30 μM) and the adenosine A_3-receptor agonist N^6-2-(4-aminophenyl)ethyl-adenosine (APNEA; 0.03–3 μM) caused no change. Of the nucleotides tested, ATP (30–300 μM), adenosine-5'-O-(3-thiotriphosphate) (ATPγS; 30–300 μM), adenosine-5'-O-(2-thiodiphosphate) (ADPβS; 30–300 μM), P_1,P_4-di(adenosine-5')-tetraphosphate (Ap_4A; 30–300 μM) and the preferential P_{2Y}-purinoceptor agonist, 2-methylthio-ATP (300 μM) decreased the evoked overflow of tritium. The P_{2X}-purinoceptor agonist, α,β-methylene-ATP (3–300 μM) caused no change.

3 The A_1-selective antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX; 10 nM) attenuated the effects of the nucleosides CPA (apparent pK_B value 9.8) and NECA as well as of the nucleotides ATP (apparent pK_B 9.3), ATPγS (apparent pK_B 9.2) and ADPβS (apparent pK_B 8.7). CGS-21680 and APNEA were ineffective also in the presence of DPCPX. The A_2-selective antagonist 1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (KF-17837) reduced the effects of CPA, NECA and ATPγS only when given at a concentration of 300 nM but not at 10 nM.

4 The P_2-purinoceptor antagonists, suramin (300 μM), reactive blue 2 (30 μM) and cibacron blue 3GA (30 μM) did not change the effect of CPA. Suramin and cibacron blue 3GA shifted the concentration-response curve of ATPγS to the right (apparent pK_B values 3.7 and 5.0, respectively). Reactive blue 2 also attenuated the effect of ATPγS, and cibacron blue 3GA attenuated the effect of ATP, but in these cases the agonist concentration-response curves were not shifted to the right. There was no antagonistic effect of suramin against ATP and ADPβS.

5 The results indicate that rat cerebrocortical noradrenergic axons possess, in addition to the known adenosine A_1-receptor, a separate purinoceptor for nucleotides (P_2) which, in contrast to the A_1-receptor, is blocked by suramin, reactive blue 2 and cibacron blue 3GA. Nucleotides such as ATP and ATPγS activate both receptors. Inconsistencies in antagonist effects against nucleotides are probably due to this activation of two receptors. The presynaptic P_2-purinoceptor is P_{2Y}-like, as it is in the peripheral sympathetic nervous system.

Keywords: Rat brain cortex; P_1-purinoceptor; P_2-purinoceptor; presynaptic purinoceptors; noradrenaline release; adenine nucleotides; adenosine 5'-O-(3-thiotriphosphate) (ATPγS); suramin; reactive blue 2; cibacron blue 3GA

Introduction

ATP inhibits the release of noradrenaline in dog subcutaneous adipose tissue (Fredholm, 1974), and adenosine inhibits the release of noradrenaline in rabbit kidney, guinea-pig vas deferens and, again, dog subcutaneous adipose tissue (Hedqvist & Fredholm, 1976). Since these initial observations, our knowledge of the presynaptic modulation of noradrenaline release by nucleosides and nucleotides has become complex, for two main reasons. First, nucleotides can act both as such and after dephosphorylation. Second, there are several presynaptic sites of action: (i) Presynaptic inhibition through adenosine receptors (P_1-purinoceptors) of the A_1 subtype is widespread (see Stone, 1981; Fredholm & Dunwiddie, 1988). Nucleotides also may produce inhibition through these receptors, both directly (Lukacsko & Blumberg, 1982; von Kügelgen *et al.*, 1989; 1992a; Fuder & Muth, 1993; Kurz *et al.*, 1993) and after breakdown to adenosine (e.g., De Mey *et al.*, 1979). (ii) Some noradrenergic terminal axons possess release-enhancing adenosine A_2- in addition to release-inhibiting A_1-receptors (Fuder *et al.*, 1992; Gonçalves & Queiroz, 1993; Rensing *et al.*, 1993). (iii) Presynaptic P_2-purinoceptors, sensitive to nucleotides but not nucleosides, are a third group, found at postganglionic sympathetic axons and mediating mainly inhibition (von Kügelgen *et al.*, 1989; 1993; 1994a; Fuder & Muth, 1993; Kurz *et al.*, 1993; Allgaier *et al.*, 1994; see also Stjärne & Åstrand, 1985), although facilitation of noradrenaline release has also been described (Miyahara & Suzuki, 1987; Sperlagh & Vizi, 1991; Allgaier *et al.*, 1994). (iv) As an alternative it has been suggested that adenosine as well as adenine nucleotides inhibit the release of noradrenaline at a common, novel, presynapatic purinoceptor that the authors call P_3 (Shinozuka *et al.*, 1988; Forsyth *et al.*, 1991; Todorov *et al.*, 1994).

To our knowledge, presynaptic P_2-purinoceptors have never been identified at central noradrenergic (or non-noradrenergic) neurones, despite studies with P_2-selective agonists (Stone & Cusack, 1989; von Kügelgen *et al.*, 1992a; for review see Hoyle & Burnstock, 1991). In a previous investigation we observed that the release of noradrenaline in brain cortex slices from rabbits was reduced through A_1- but

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not changed through P_2-purinoceptors (von Kügelgen et al., 1992a). We have now carried out an analogous search in rats. Previous results suggest that rat cerebrocortical noradrenergic axons possess A_1-receptors (Harms et al., 1978; Craig & White, 1992). Reasons for the choice of the rat were the operation of P_2-purinoceptors at peripheral noradrenergic axons of this species (Fuder & Muth, 1993; Kurz et al., 1993; von Kügelgen et al., 1994a) as well as the cell bodies of the cerebrocortical noradrenergic neurones in the locus coeruleus (Tschöpl et al., 1992; Shen & North, 1993). Slices of rat brain cortex were preincubated with [^{3}H]-noradrenaline, and transmitter release was elicited by electric stimulation. Some of these results have been published in abstract form (von Kügelgen et al., 1994b).

Methods

Male Wistar rats weighing 250–300 g (Savo, Kisslegg, Germany) were killed by cervical dislocation and exsanguination. The brain was quickly removed and chilled. Two slabs were cut from the occipito-parietal cortex, parallel to the surface. The superficial slab, 0.2 mm thick, was discarded and round slices of 3.5 m diameter were punched from the second slab, 0.3 mm thick. Six to eight slices were preincubated at 37°C for 30 min in 2 ml medium containing (−)-[^{3}H]-noradrenaline, 0.1 μM. One slice was then transferred to each of six 0.16-ml superfusion chambers where it was held by a polypropylene mesh between platinum wire electrodes, 6 mm apart. The slices were superfused with [^{3}H]-noradrenaline-free medium for 107 min at a rate of 0.6 ml min^{-1} at 37°C. A Stimulator I (Hugo Sachs Elektronik, March-Hugstetten, Germany) operating in the constant voltage mode was used for electrical field stimulation. Three periods of stimulation were applied (rectangular pulses of 2 ms width and 12.5 V cm^{-1}, yielding a current strength of 18 mA). The first stimulation period was delivered after 20 min of superfusion and consisted of 18 pulses at 1 Hz. It was not used for determination of tritium overflow. The following two periods were delivered after 59 (S_1) and 89 (S_2) min of superfusion and consisted of a train of either 4 pulses/100 Hz or 60 pulses/1 Hz (identical parameters at S_1 and S_2 in each single experiment); unless stated otherwise, the parameters were 4 pulses/100 Hz. The collection of successive 3-min superfusate samples began 9 min before S_1. Some drugs (or solvents) were present in the medium throughout superfusion. Other drugs (or solvents) were added before S_2 (6 min before S_2 unless stated otherwise) and kept for the remainder of the experiment; the delay from addition to medium to arrival at tissue was about 60 s. After superfusion, each slice was solubilized in 0.5 ml Soluene-350 (Canberra Packard, Frankfurt am Main, Germany). Tritium was measured in superfusate samples and solubilized slices by liquid scintillation counting.

The medium used for incubation and superfusion contained (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03. It was saturated with 5% CO_2 in O_2. The pH was adjusted to 7.4 with NaOH 1 M. The medium used for superfusion (but not that used for preincubation) contained desipramine 1 μM in order to block uptake$_1$.

The outflow of tritium was expressed as a fractional rate (min^{-1}) and the evoked overflow, obtained by subtraction of the estimated basal outflow, as a percentage of the tritium content of the slice (von Kügelgen et al., 1992a). For further evaluation of basal tritium efflux, ratios were calculated of the fractional rate of outflow in the 3-min interval before S_2 and in the 3-min interval before S_1 (b_2/b_1). For further evaluation of the electrically evoked overflow, ratios were calculated of the overflow elicited by S_2 and the overflow elicited by S_1 (S_2/S_1). S_2/S_1 ratios obtained in individual experiments with a test compound A added before S_2 were calculated as a percentage of the mean S_2/S_1 ratio in the appropriate control group (solvent instead of A). When the interaction of A, added before S_2, and a drug B, added throughout superfusion, was studied, the 'appropriate control' was a group in which B alone was used.

The following drugs were used: suramin hexasodium salt (Bayer, Wuppertal, Germany); (−)-[ring-2,5,6-^{3}H]-noradrenaline, specific activity 1.50 to 2.11 TBq mmol^{-1} (Du Pont, Dreieich, Germany); N^6-2-(4-aminophenyl)ethyl-adenosine (APNEA) (Prof. R.A. Olsson, Dept. of Internal Medicine, University of South Florida, Tampa, Florida, U.S.A.); 2-*p*-(2-carbonylethyl)-phenethylamino-5′-N-ethylcarboxamido-adenosine HCl (CGS-21680), N^6-cyclopentyl-adenosine (CPA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-methylthio-adenosine-5′-triphosphate tetrasodium salt (2-methylthio-ATP), reactive blue 2 (R-115 in RBI catalogue 1992-93; Colour Index No. 61211 in Society of Dyers and Colourists, 1971) (Research Biochemicals, Biotrend, Köln, Germany); yohimbine HCl (Roth, Karlsruhe, Germany); adenosine-5′-O-(2-thiodiphosphate) lithium salt (ADPβS), adenosine-5′-O-(3-thiotriphosphate) lithium salt (ATPγS), ATP disodium salt, cibacron blue 3GA (C-9534 in Sigma catalogue 1993; isomer of reactive blue 2 in which the sulphonic acid residue at the terminal benzene ring is in the *o*-position; see footnote on page 130 of von Kügelgen et al., 1994a), desipramine HCl, P_1,P_4-di(adenosine-5′)-tetraphosphate ammonium salt (Ap_4A), 5′-N-ethylcarboxamido-adenosine (NECA), indomethacin, α,β-methylene-adenosine-5′-diphosphate (α,β-methylene-ADP, APCP), α,β-methylene-adenosine-5′-triphosphate lithium salt (α,β-methylene-ATP, APCPP), N^G-nitro-L-arginine, tetrodotoxin (Sigma, Deisenhofen, Germany); 1,3-dipropyl-8-(3,4-dimethoxystyryl)-7-methylxanthine (KF-17837) (Dr F. Suzuki, Kyowa Hakko Kogyo, Pharmaceutical Research Laboratories, Sunto-Gun, Shizuoka-Ken, Japan). Solutions of drugs were prepared with distilled water, or (indomethacin) the KH_2PO_4- and $NaHCO_3$- containing stock solution of the medium, or (APNEA, CGS-21680, DPCPX, KF-17837) dimethyl sulphoxide (final concentration about 0.4 mM), or (CPA) ethanol (final concentration about 3 mM), or (tetrodotoxin) sodium acetate buffer (0.1 M, pH 4.8), or (NECA) tartaric acid (final concentration about 0.1 mM). Solutions of KF-17837 were protected from direct sunlight. Appropriate amounts of the solvents given before S_2 did not change basal tritium efflux or the evoked overflow. Dimethyl sulphoxide 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 brain slices.

Results

Electrical stimulation by 4 pulses/100 Hz or 60 pulses/1 Hz sharply increased the outflow of tritium from rat brain cortex slices preincubated with [^{3}H]-noradrenaline (as in Figure 1 of Trendelenburg et al., 1993). In the presence of desipramine (1 μM) alone, which was always present throughout superfusion, the overflow elicited by 4 pulses/100 Hz at S_1 averaged 1.641 ± 0.025% of the tritium content of the tissue (corresponding to 96.8 ± 1.6 Bq; b_1, i.e. basal tritium efflux before S_1 was 0.00364 ± 0.00004 min^{-1}; $n = 323$), and the overflow elicited by 60 pulses/1 Hz at S_1 averaged 2.843 ± 0.081% of tissue tritium (b_1 0.00353 ± 0.00013 min^{-1}; $n = 30$). When no additional drug was administered between S_1 and S_2, b_2, i.e. the basal overflow of tritium before S_2, was slightly lower than b_1, and the outflow elicited by S_2 was similar to the overflow at S_1. For example, the control b_2/b_1 ratio for experiments with 4 pulses/100 Hz was 0.91 ± 0.05, and the control S_2/S_1 ratio was 1.02 ± 0.01 (desipramine alone present throughout superfusion; $n = 66$). Trains of 4

pulses/100 Hz were used except in experiments described at the end of the Results section.

Effects of adenine nucleosides and nucleotides

When added 6 min before S_2, the adenine nucleosides and nucleotides tested did not change the basal efflux of tritium (b_2/b_1) except for a slight decrease by CPA 300 nM and ATP 300 μM and a slight increase by Ap_4A 300 μM. As shown in Figure 1a, the selective adenosine A_1-receptor agonist CPA as well as the non-subtype-selective agonist NECA (Williams *et al.*, 1986) reduced the evoked overflow of tritium by about 30% maximally (no true maximum reached in the case of NECA). The selective adenosine A_{2a}-receptor agonist CGS-21680 (Jarvis *et al.*, 1989) and the preferential adenosine A_3-receptor agonist APNEA (Zhou *et al.*, 1992) caused no change (Figure 1a). Of the nucleotides examined, ATP, ATPγS, ADPβS, Ap_4A and the preferential P_{2Y}-purinoceptor agonist 2-methylthio-ATP (Kennedy, 1990; Hoyle & Burnstock, 1991) reduced the evoked overflow (Figure 1b). ATP and its metabolically more stable analogue ATPγS (Welford *et al.*, 1986) were equipotent and at the highest concentration tested, 300 μM, produced greater inhibition (by about 45%) than any other compound of Figure 1. The preferential

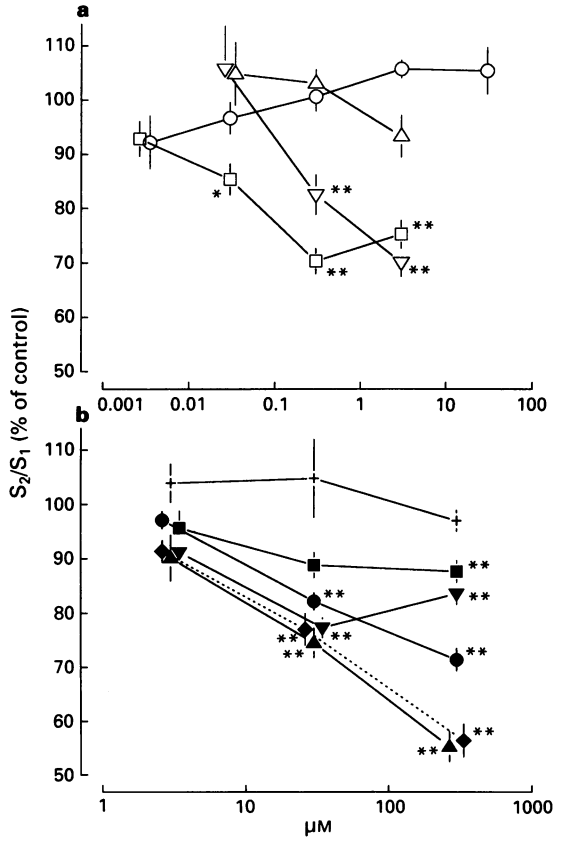

Figure 1 Effects of adenosine analogues (a) and adenine nucleotides (b) on electrically evoked tritium overflow from rat brain cortex slices preincubated with [^{3}H]-noradrenaline. Slices were stimulated twice by 4 pulses/100 Hz (S_1, S_2). N^6-2-(4-aminophenyl)ethyl-adenosine (APNEA, Δ), 2-*p*-(2-carbonylethyl)-phenethylamino-5'-N-ethylcarboxamido-adenosine (CGS-21680, ○), N^6-cyclopentyl-adenosine (CPA, □), 5'-N-ethylcarboxamido-adenosine (NECA, ▽), adenosine 5'-O-(2-thiodiphosphate) (ADPβS, ●), adenosine 5'-O-(3-thiotriphosphate) (ATPγS, ◆), ATP (▲), P_1, P_4-di(adenosine-5')-tetraphosphate (Ap_4A, ▼), α,β-methylene-ATP (+) or 2-methylthio-ATP (■) was added 6 min before S_2 for the remainder of the experiment. Ordinates, evoked tritium overflow: S_2/S_1 ratio obtained in individual tissue slices were calculated as a percentage of the average control (solvent) S_2/S_1 ratio. Means ± s.e.mean of 4–12 experiments. Significant differences from control: $^*P<0.05$ and $^{**}P<0.01$.

P_{2X}-purinoceptor agonist α,β-methylene-ATP (Kennedy, 1990; Hoyle & Burnstock, 1991) caused no change.

ATPγS 30 μM produced a similar decrease, irrespective of whether it was added 6 min (Figure 1b) or 12 min before S_2 (not shown; $n = 4$ for the 12-min experiment).

Interactions

Drugs tested for their interaction with nucleosides and nucleotides were added throughout superfusion (in addition to desipramine). They caused only minimal changes of the basal efflux of tritium (b_1), and only cibacron blue 3GA 30 μM caused a significant change (an increase by 21%) of the overflow evoked by S_1 (not shown). Nucleosides, nucleotides or their solvents were added before S_2. When solvent was administered before S_2, the b_2/b_1 ratio again was slightly below, and the S_2/S_1 ratio close to, unity (not shown).

We first tested the interaction with P_1-purinoceptor antagonists. DPCPX selectively blocks adenosine A_1-receptors (Bruns *et al.*, 1987; Lohse *et al.*, 1987). DPCPX 10 nM shifted the concentration-response curves of CPA, ADPβS, ATP and ATPγS to the right by similar degrees (Figure 2). From the shifts at the level of 15% inhibition, apaprent pK_B values of DPCPX against CPA, ADPβS, ATP and ATPγS of 9.8, 8.7, 9.3 and 9.2, respectively, were calculated (equation No. 4 of Furchgott, 1972). DPCPX 10 nM abolished the inhibition by NECA 0.3 and 3 μM but did not reverse it to an increase in evoked tritium overflow ($n = 6$ and 4, respectively; not shown). CGS-21680 0.3 and 30 μM and APNEA 3 μM were ineffective in the presence of DPCPX ($n = 4$ or 5; not shown) as they had been in the absence of the A_1 antagonist (Figure 1a).

KF-17837 is a P_1-purinoceptor antagonist which preferentially blocks the adenosine A_2-receptor (Shimada *et al.*, 1992; Jackson *et al.*, 1993). At a concentration of 10 nM, it failed to antagonize CPA 0.3 μM, NECA 0.3 μM and ATPγS 30 μM ($n = 4–7$; not shown). KF-17837 300 nM, however, significantly attenuated the effects of CPA 0.3 μM (S_2/S_1 ratio 85.4 ± 6.8% of control; $n = 6$) and ATPγS 30 μM (S_2/S_1 90.5 ± 4.5% of control; $n = 6$) and abolished the effect of NECA 0.3 μM on the evoked overflow of tritium ($n = 5$; not shown).

Antagonists at P_2-purinoceptors were the second group of drugs studied for their interaction with nucleosides and nucleotides. The non-subtype-selective P_2 antagonist, suramin (Dunn & Blakeley, 1988), when added throughout superfusion at a concentration of 300 μM, did not change the inhibitory effects of CPA, ADPβS and ATP but caused a small shift to the right of the concentration-response curve of ATPγS (Figure 3). The shift at the level of 25% inhibition corresponded to an apparent pK_B value of suramin against ATPγS of 3.7.

Reactive blue 2 is a preferential antagonist at P_{2Y}-purinoceptors (Burnstock & Warland, 1987; Houston *et al.*, 1987). Reactive blue 30 μM did not change the inhibition by CPA but attenuated the inhibition by ATPγS, 300 μM (Figure 4).

Selective antagonism against nucleotides was also observed with cibacron blue 3GA, an isomer of reactive blue 2 and also P_{2Y}-selective (e.g., Shirahase *et al.*, 1991; Boland *et al.*, 1992; for the nomenclature of reactive blue 2 and cibacron blue 3GA see footnote on p. 130 of von Kügelgen *et al.*, 1994a). Cibacron blue 3GA 30 μM left the effect of CPA unchanged, tended to reduce the effect of ADPβS, reduced significantly the effect of ATP 300 μM, and shifted the concentration-response curve of ATPγS to the right (Figure 5). The shift at the level of 25% inhibition corresponded to an apparent pK_B value of cibacron blue 3GA against ATPγS of 5.0.

The combination of cibacron blue 3GA (30 μM) with DPCPX (10 nM) was also tested. The mixture shifted the concentration-response curve of ATPγS beyond the antagonism caused by DPCPX alone (Figure 2d) and cibacron blue 3GA alone (Figure 5d). The shift beyond that produced

by DPCPX alone (Figure 2d), when read at the level of 10% inhibition, yielded an apparent pK_B for cibacron blue 3GA of 5.1, similar to the 5.0 value obtained in the absence of DPCPX (preceding paragraph).

In search for possible mediators of the inhibition by adenine nucleotides, ATPγS was administered in the combined presence of indomethacin and N^G-nitro-L-arginine which were added throughout superfusion at concentrations (10 μM each) known to block the synthesis of prostaglandins (Starke *et al.*, 1977) and nitric oxide (Knowles *et al.*, 1990), respectively. The inhibition by ATPγS 30 μM remained unchanged ($n = 5$; not shown).

The inhibitor of 5'-nucleotidase α,β-methylene-ADP, given throughout superfusion at a concentration (100 μM) blocking the breakdown of AMP to adenosine (Bruns, 1980; see also Cunha *et al.*, 1994), did not change the effect of ATPγS 30 and 300 μM ($n = 6$ and 7; not shown). When both α,β-methylene-ADP and cibacron blue 3GA 30 μM were present throughout superfusion, the concentration-response curve of ATPγS was shifted to the right to the same extent (see Figure 5d) as by cibacron blue 3GA alone ($n = 5$ per ATPγS concentration; not shown).

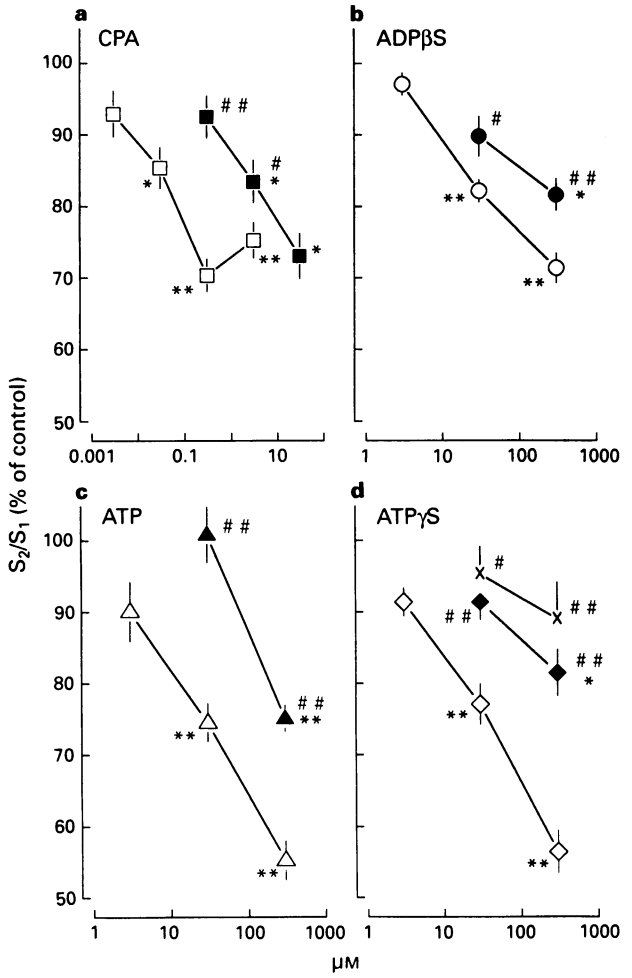

Figure 2 Interaction of purinoceptor agonists with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or cibacron blue 3GA combined with DPCPX. Slices were stimulated twice by 4 pulses/100 Hz (S_1, S_2).N^6-cyclopentyl-adenosine (CPA, a), adenosine 5'-O-(2-thiodiphosphate) (ADPβS, b), ATP (c) or adenosine 5'-O-(3-thiotriphosphate)(ATPγS, d) was added 6 min before S_2 for the remainder of the experiment. Open symbols represent experiments in which CPA, ADPβS, ATP orATPγS was given alone (taken from Figure 1). Solid symbols represent experiments in which the medium contained DPCPX 10 nM throughout superfusion; (x) represents experiments in which the medium contained both cibacron blue 3GA 30 μM and DPCPX 10 nM throughout superfusion (d). Ordinates, evoked tritium overflow: S_2/S_1 ratios obtained in individual tissue slices were calculated as a percentage of the average S_2/S_1 ratio in the appropriate control group (solvent instead of agonists). Means ± s.e.mean of 4–12 experiments. Significant differences from corresponding control: $*P<0.05$ and $**P<0.01$. Significant differences from experiments in which CPA, ADPβS, ATP or ATPγS was given alone: $\#P<0.05$ and $\#\#P<0.01$.

Effects of purinoceptor antagonists, tetrodotoxin and yohimbine

When present throughout superfusion, the purinoceptor antagonists did not change the overflow of tritium evoked by 4 pulses/100 Hz (S_1) except for a small increase by cibacron blue 3GA 30 μM (see above). Drug effects on the electrically evoked overflow of tritium in this kind of experiment are assessed more accurately when the drugs are given before S_2 so that S_1 is a reference value for each brain slice. However, DPCPX 10 nM, suramin 300 μM and reactive blue 2 30 μM, also caused no change in evoked tritium overflow when applied before S_2, and the same was true for cibacron blue 3GA 30 μM. Two drugs unrelated to purines were administered: tetrodotoxin 0.3 μM abolished the overflow response to 4 pulses/100 Hz; yohimbine 1 μM was ineffective (Table 1).

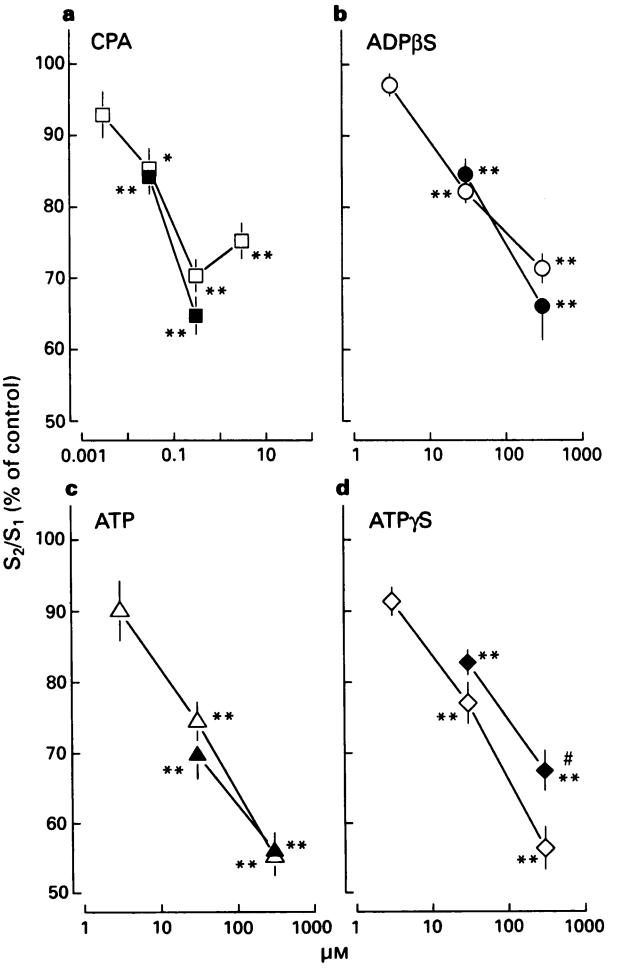

Figure 3 Interaction of purinoceptor agonists with suramin. Slices were stimulated twice by 4 pulses/100 Hz (S_1, S_2). N^6-cyclopentyl-adenosine (CPA, a), adenosine 5'-O-(2-thiodiphosphate) (ADPβS, b), ATP (c) or adenosine 5'-O-(3-thiotriphosphate) (ATPγS, d) was added 6 min before S_2 for the remainder of the experiment. Open symbols represent experiments in which CPA, ADPβS, ATP or ATPγS was given alone. Solid symbols represent experiments in which the medium contained suramin 300 μM throughout superfusion. Means ± s.e.mean of 5–14 experiments. Other details as in Figure 2.

Slices were stimulated by 60 pulses/1 Hz instead of 4 pulses/100 Hz in a final series of experiments. The purinoceptor antagonists again caused no change, whereas yohimbine now increased the evoked overflow of tritium 2.4 fold (Table 1).

Discussion

Under the conditions of the present experiments, the overflow of tritium, elicited by electrical stimulation in the presence of desipramine, reflects a quasi-physiological release of [^{3}H]-noradrenaline (see Taube *et al.*, 1977). The release evoked by 4 pulses/100 Hz was free from presynaptic α_2-autoinhibition as shown by the lack of effect of yohimbine (cf. Zier *et al.*, 1988). Hence, any potential interaction between presynaptic α_2-adrenoceptor and purinoceptor mechanisms was avoided.

Presynaptic adenosine A_1-receptors

Our experiments confirm the operation of release-inhibiting A_1-receptors at the noradreneregic terminal axons in rat brain cortex (Harms *et al.*, 1978; Craig & White, 1992) and indicate the absence of additional P_1-purinoceptor subtypes. In accord with this view, only the A_1-receptor agonist CPA and the non-subtype-selective agonist NECA, but neither the A_{2a}-receptor agonist CGS-21680 nor the A_3-receptor agonist APNEA altered the evoked overflow of tritium. CPA was about 8 times more potent than NECA in causing inhibition (Figure 1a), as it is at A_1 radioligand binding sites in rat brain membranes (Williams *et al.*, 1986). The adenosine A_1-receptor antagonist DPCPX shifted the concentration-response curve of CPA to the right with an apparent pK_B value (9.8) close to values found at presynaptic A_1-receptors in other rat tissues (9.3–9.7; Sebastião *et al.*, 1990; Fuder *et al.*, 1992; Kurz *et al.*, 1993). DPCPX also blocked the effect of NECA. On the other hand, the preferential adenosine A_2-receptor antagonist KF-17837 attenuated the effects of

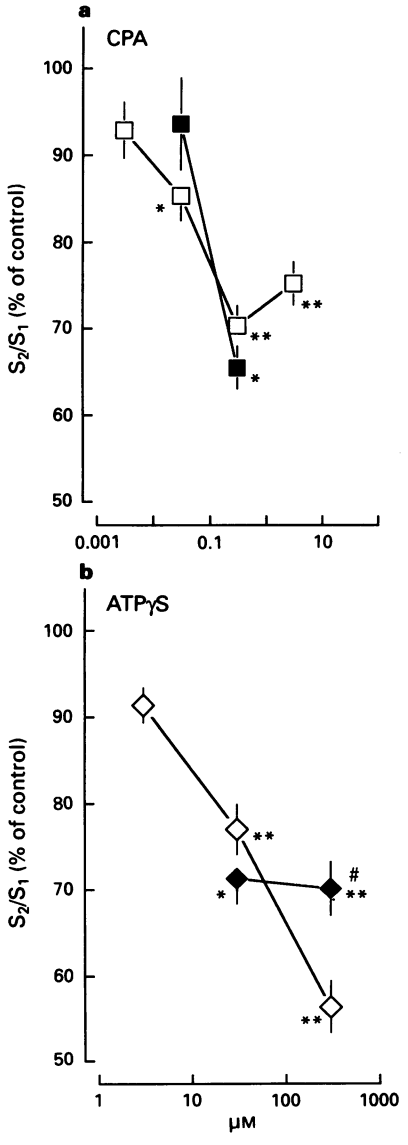

Figure 4 Interaction of purinoceptor agonists with reactive blue 2. Slices were stimulated twice by 4 pulses/100 Hz (S_1, S_2). N^6-cyclopentyl-adenosine (CPA, a) or adenosine 5′-O-(3-thiotriphosphate) (ATPγS, b) was added 6 min before S_2 for the remainder of the experiment. Open symbols represent experiments in which CPA or ATPγS was given alone. Solid symbols represent experiments in which the medium contained reactive blue 2 30 μM throughout the superfusion. Means ± s.e.mean of 5–12 experiments. Other details as in Figure 2.

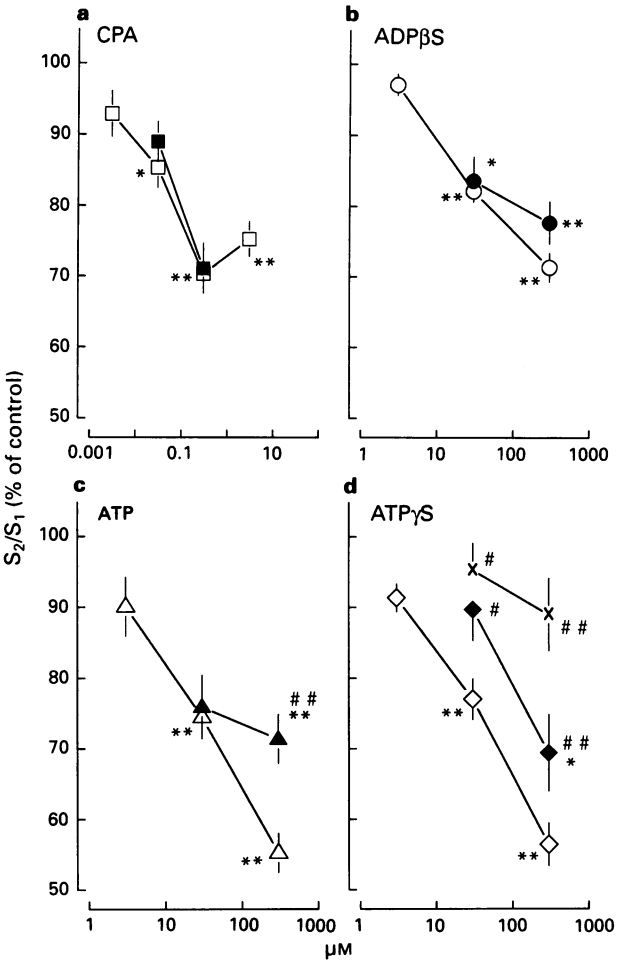

Figure 5 Interaction of purinoceptor agonists with cibacron blue 3GA or cibacron blue 3GA combined with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). Slices were stimulated twice by 4 pulses/100 Hz (S_1, S_2). N^6-cyclopentyl-adenosine (CPA, a) adenosine 5′-O-(2-thiodiphosphate) (ADPβS, b), ATP (c) or adenosine 5′-O-(3-thiotriphosphate) (ATPγS, d) was added 6 min before S_2 for the remainder of the experiment. Open symbols represent experiments in which CPA, ADPβS, ATP or ATPγS was given alone. Solid symbols represent experiments in which the medium contained cibacron blue 3GA 30 μM throughout superfusion; (x) represents experiments in which the medium contained both cibacron blue 3GA 30 μM and DPCPX 10 nM throughout superfusion (d; identical with (x) in Figure 2d). Means ± s.e.mean of 4–12 experiments. Other details as in Figure 2.

Table 1 Effects of purinoceptor antagonists, yohimbine and tetrodotoxin on electrically evoked tritium overflow

Drugs added 6 min before S_2	*S_2/S_1 (% of control) 4 pulses/100 Hz*	*n*	*60 pulses/1 Hz*	*n*
–	100.0 ± 1.2	10	100.0 ± 1.5	6
DPCPX (10 nM)	103.5 ± 1.9	6	109.4 ± 3.1	5
Suramin (300 μM)	101.1 ± 1.8	4	103.2 ± 2.0	5
Reactive blue 2 (30 μM)	95.0 ± 1.7	6	110.8 ± 7.6	5
Cibacron blue 3GA (30 μM)	107.1 ± 3.0	7	111.9 ± 5.6	5
Tetrodotoxin (0.3 μM)	3.1 ± 1.3**	6	–	
Yohimbine (1 μM)	97.8 ± 8.2	6	240.9 ± 14.4*	4

Slices were stimulated twice (S_1, S_2; pulse number and frequency indicated). Drugs or solvent were added 6 min before S_2 for the remainder of the experiment. S_2/S_1 ratios obtained in individual tissue slices were calculated as a percentage of the average control (solvent) S_2/S_1 ratio. DPCPX, 8-cyclopentyl-1,3-dipropylxanthine. Means ± s.e. mean of *n* experiments.
Significant differences from corresponding control: $^*P<0.05$ and $^{**}P<0.01$.

CPA and NECA only at the high concentration of 300 nM, close to its K_i for inhibition of radioligand binding to A_1-receptors in guinea-pig brain membranes (Shimada *et al.*, 1992). Finally, none of the agonists increased the evoked overflow of tritium, not even in the presence of DPCPX when a release-enhancing non-A_1 effect might have been unmasked (Brown *et al.*, 1990; Rensing *et al.*, 1993). The absence of release-enhancing A_2-purinoceptors contrasts with some other noradrenergic neurone systems (see Introduction).

The cerebrocortical presynaptic A_1-purinoceptors were sites of action not only of the nucleosides CPA and NECA. DPCPX shifted the concentration-inhibition curves of ADPβS, ATP and ATPγS to the right with apparent pK_B values (8.7 to 9.3) similar to the pK_B against CPA (9.8) and similar to values at presynaptic A_1-receptors in other rat tissues (9.3–9.7; see above). Blockade of 5'-nucleotidase by α,β-methylene-ADP did not attenuate the effect of ATPγS, indicating that breakdown of nucleotides to adenosine was not necessary for the inhibition. It has been suggested that adenosine and adenine nucleotides activate a common presynaptic receptor in some sympathetically innervated peripheral tissues and that this receptor is a novel subtype, P_3 (Shinozuka *et al.*, 1988; Forsyth *et al.*, 1991; Todorov *et al.*, 1994). While our results support the notion of a common presynaptic nucleoside and nucleotide receptor, the potent antagonist effect of DPCPX identifies the receptor in rat brain cortex as A_1. In this, the rat brain cortex noradrenergic axons would agree with the noradrenergic axons of rabbit brain cortex, rat vas deferens and mouse vas deferens (von Kügelgen *et al.*, 1992a; 1994a; Fuder & Muth, 1993; Kurz *et al.*, 1993; compare the studies on non-noradrenergic neurones by Moody *et al.*, 1984; Wiklund *et al.*, 1985; Rubino *et al.*, 1992; Cunha *et al.*, 1994). Adenine nucleotides also activate A_1-receptors in non-neural cells (Bailey *et al.*, 1992). One major component of the presynaptic inhibition by adenine nucleotides was, hence, A_1-mediated.

Presynaptic P_{2Y}-like purinoceptors

The nucleotides depressed the release of noradrenaline through a second site, a P_2-purinoceptor. One piece of evidence is the inhibition produced by 2-methylthio-ATP, a nucleotide selective for the P_{2Y}-subtype P_2-purinoceptor (Kennedy, 1990; Hoyle & Burnstock, 1991) and inactive at A_1-receptors (Tschöpl *et al.*, 1992; von Kügelgen *et al.*, 1992a). A more important piece of evidence is the observation that three P_2-purinoceptor antagonists, suramin, reactive blue 2 and cibacron blue 3GA, while not changing the effect of CPA, tended to attenuate or attenuated significantly the effect of the nucleotides, most consistently that of ATPγS (Figures 3–5).

Cibacron blue 3GA shifted the concentration-response curve of ATPγS to the right with an apparent pK_B value (5.0) similar to that found at the P_2-purinoceptors of the sympathetic axons of rat iris (4.7; Fuder & Muth, 1993). Virtually the same apparent pK_B (5.1) was obtained when the concentration-response curve of ATPγS in the presence of cibacron blue 3GA plus DPCPX was compared with the curve determined in the presence of DPCPX alone (Figure 2d); if DPCPX and cibacron blue 3GA had blocked the same receptor, the combination should have caused a much smaller shift beyond that caused by DPCPX alone (cf. Kurz *et al.*, 1993). The failure of indomethacin and N^G-nitro-L-arginine to attenuate the inhibition by ATPγS, excludes prostaglandins or nitric oxide as mediators of the effect of the nucleotide. Although alternatives are hard to exclude (see Starke, 1981), it seems most likely that the P_2-purinoceptors are located on the noradrenergic terminal axons themselves. Release-inhibiting P_2-purinoceptors have previously been demonstrated at the postganglionic sympathetic axons of mouse and rat vas deferens (von Kügelgen *et al.*, 1989; 1993; 1994a; Kurz *et al.*, 1993) and rat iris (Fuder & Muth, 1993) and at cultured chick postganglionic sympathetic neurones (Allgaier *et al.*, 1994). This is the first report of P_2-purinoceptors modulating transmitter release in the CNS.

One may ask why cibacron blue 3GA antagonized the effect of ATPγS more consistently than effects of ADPβS and ATP (Figure 5); why suramin antagonized only ATPγS but not ADPβS and ATP (Figure 3); why the antagonism of cibacron blue 3GA against ATP (Figure 5c) and of reactive blue 2 against ATPγS (Figure 4b) differed so markedly from a rightward shift; and why the apparent pK_B value of suramin against ATPγS (3.7) was lower than at other P_2-purinoceptors (for example 4.7 and 5.0 at P_{2X}- and P_{2Y}-purinoceptors in guinea-pig urinary bladder and taenia coli, respectively; Hoyle *et al.*, 1990). The likely common reason is the action of all nucleotides at two receptors, A_1 and P_2, a fact that leads to deviations from the rules valid for one-receptor systems (see p. 298 of Furchgott, 1972). ATPγS may have relied on the P_2 component to a greater extent than ADPβS and ATP and, hence, may have been more susceptible to P_2 antagonists, as it was at the presynaptic P_2-purinoceptors in mouse van deferens (von Kügelgen *et al.*, 1989). It should be noted that the apparent pK_B values of DPCPX against the nucleotides (8.7–9.3) also were slightly lower than the pK_B against CPA (9.8), possibly for the same reason, as suggested previously by Fuder & Muth (1993). Generally speaking, the 'apparent pK_B values' of the present study must be considered as rough affinity estimates, because of the two-receptor situation, because curve shifts often were small, and because only one antagonist concentration and few agonist concentrations were used.

The involvement of two receptors in the effect of nucleotides impedes the subclassification of the presynaptic P_2-purinoceptor. Nevertheless, the P_2-purinoceptor at postganglionic sympathetic neurones has been described as P_{2Y}-like (von Kügelgen *et al.*, 1989; 1994a; Fuder & Muth, 1993; Kurz *et al.*, 1993). The effect of 2-methylthio-ATP, combined with lack of effect of α,β-methylene-ATP, makes the presynaptic P_2-purinoceptor in rat brain cortex P_{2Y}-like as well. The antagonism by reactive blue 2 and cibacron blue 3GA is in accord with this view: both are P_{2Y}-selective and have previously been shown to block the presynaptic P_{2Y}-like purinoceptors in mouse vas deferens (von Kügelgen *et al.*, 1994a) and rat iris (Fuder & Muth, 1993). The rat cerebrocortical presynaptic P_2-purinoceptor differs, however, from classical P_{2Y}-purinoceptors at which 2-methylthio-ATP is known to act at nanomolar concentrations (see Fischer *et al.*, 1993). It also differs from the P_2-purinoceptor at the cell bodies of the noradrenergic neurones in the rat locus coeruleus, which mediates an increase in firing rate and

which has equally been classified as P_{2Y}-like (Illes & Nörenberg, 1993; Shen & North, 1993): in the locus coeruleus, 2-methylthio-ATP and α,β-methylene-ATP were about equipotent and more potent than ATP. The existence of several kinds of P_{2Y}-purinoceptor has recently been confirmed by molecular cloning (see Barnard *et al.*, 1994).

An endogenous input?

Presynaptic A_1-receptors are potential sites of action of endogenous adenosine (as well as adenine nucleotides; see above), and presynaptic P_{2Y}-like receptors are potential sites of action of endogenous ATP. Under the conditions of the present experiments, however, the receptors did not play this 'physiological' role: DPCPX and the P_2 antagonists caused no consistent increase in the release of [^{3}H]-noradrenaline, neither when the pulse trains were very brief (4 pulses/100 Hz) nor when they were longer (60 pulses/1 Hz) (cf. Harms *et al.*, 1978). Presynaptic α_2-autoinhibition, in contrast, depressed transmitter release during the 60 pulses/1 Hz trains, as shown by the effect of yohimbine (Table 1) and as expected (see Starke *et al.*, 1989). The results, of course, do not exclude an endogenous input to the presynaptic purinoceptors under other conditions. The release of noradrenaline is depressed by endogenous A_1-agonists in several tissues such as rat (Jonzon & Fredholm, 1984) and rabbit hippocampus (Jackisch *et al.*, 1985) and rabbit brain cortex (von Kügelgen *et al.*, 1992a,b). An endogenous agonist input to presynaptic P_2-purinoceptors has recently been demonstrated for the postganglionic sympathetic nerves of the mouse, rat and rabbit vas deferens (von Kügelgen *et al.*, 1993, 1994; Grimm *et al.*, 1994).

Conclusion

The noradrenergic axons of the rat brain cortex possess two release-inhibiting purinoceptor systems, A_1 and P_2. The A_1-receptors are activated by nucleosides such as CPA and also by (certain) nucleotides such as ATPγS and are blocked by DPCPX but not by suramin, reactive blue 2 and cibacron blue 3GA. The P_2-receptors are activated by nucleotides but not by nucleosides and are blocked by suramin, reactive blue 2 and cibacron blue 3GA but not by DPCPX; they are P_{2Y}-like. Neither purinoceptor was activated by an endogenous ligand under the present experimental conditions.

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