Ionotropic glutamate receptor types leading to adenosine-mediated inhibition of electrically evoked [³H]-noradrenaline release in rabbit brain cortex slices

Ivar von Kügelgen, Leni Späth & Klaus Starke

Pharmakologisches Institut, Hermann-Herder-Strasse 5, D-79104 Freiburg i. Br., Germany

1 Glutamate inhibits the electrically evoked release of noradrenaline in rabbit brain cortex slices; the inhibition is mediated by adenyly compounds, presumably adenosine. The aim of the present study was to identify the receptors involved in this indirect inhibitory effect of glutamate. Slices of the occipitoparietal cortex were preincubated with [³H]-noradrenaline and then superfused and stimulated by trains of 6 pulses, 100 Hz.

2 The ionotropic glutamate receptor agonists α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA; 10–100 μ M), kainate (10–100 μ M) and N-methyl-D-aspartate (NMDA; 30–300 μ M) but not the metabotropic glutamate receptor agonist, 1-amino-1,3-cyclopentanedicarboxylate (ACPD; 10–100 μ M) reduced the electrically evoked overflow of tritium.

3 The effects of AMPA, kainate and NMDA were attenuated or abolished by the adenosine A₁-receptor antagonist, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) as well as by adenosine deaminase but not by the α_2 -adrenoceptor antagonist yohimbine, the γ -aminobutyric acid (GABA) receptor antagonists, bicuculline and 2-hydroxysaclofen and the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME).

4 The NMDA receptor antagonist, 2-amino-5-phosphonopentanoate (AP5) blocked the inhibitory effect of NMDA but not that of AMPA and kainate. The non-NMDA-receptor antagonist, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) blocked the effect of AMPA but not of kainate and NMDA.

5 In addition to decreasing the electrically evoked overflow of tritium, AMPA, kainate and NMDA but not ACPD caused a steep but transient rise of basal tritium efflux. This immediate releasing effect was not significantly changed by DPCPX, adenosine deaminase, yohimbine, bicuculline, 2-hydroxysaclofen and L-NAME (except that L-NAME enhanced the effect of kainate). AP5 and CNQX antagonized the immediate releasing effects in the same way that they antagonized the inhibition by AMPA, kainate and NMDA of the electrically evoked overflow of tritium.

6 It is concluded that AMPA, kainate and NMDA, like glutamate, reduce the electrically evoked release of noradrenaline by releasing adenosine or an adenine nucleotide which is then degraded to adenosine. Activation of each of the three ionotropic glutamate receptors, AMPA, kainate and NMDA receptors, but not activation of metabotropic glutamate receptors can initiate this indirect inhibitory effect on the release of noradrenaline (as well as the known noradrenaline releasing effect).

Keywords: α -Amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA); N-methyl-D-aspartate (NMDA); kainate; endogenous adenosine; ionotropic glutamate receptors; metabotropic glutamate receptors; presynaptic adenosine A₁-receptors; noradrenaline release; rabbit brain cortex

Introduction

Glutamate and other excitatory amino acids (EAA) release noradrenaline from cerebral axons (man: Fink *et al.*, 1992; rat: Nelson *et al.*, 1980; Jones *et al.*, 1987; Vezzani *et al.*, 1987; Fink *et al.*, 1989; Blandina *et al.*, 1992; Fink & Göthert, 1992; Pittaluga & Raiteri, 1992; Wang *et al.*, 1992; mouse: Zhao *et al.*, 1990). At least part of the effect is direct at the terminal noradrenergic axons since it is also observed in synaptosomal preparations (Fink & Göthert, 1992; Pittaluga & Raiteri, 1992; Wang *et al.*, 1992). It seems that activation of any of the three types of ionotropic glutamate receptor, α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA), kainate and N-methyl-D-aspartate (NMDA) receptors, will elicit the response (see references above).

We recently observed a second effect of glutamate on cerebral noradrenergic terminal axons: inhibition of the electrically evoked release of [³H]-noradrenaline (rabbit brain cortex slices; von Kügelgen *et al.*, 1992a). The effect was indirect: it was abolished by the A₁-purinoceptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) as well as by addition of the enzyme adenosine deaminase, indicating that glutamate initially released adenosine (or an adenine nucleotide which was degraded to adenosine) and adenosine then

caused presynaptic, A₁-purinoceptor-mediated inhibition (von Kügelgen *et al.*, 1992a). Release by EAA of adenyly compounds in brain cortex has been shown directly (guinea-pig: Pull & McIlwain, 1975; rat: Jhamandas & Dumbrille, 1980; Perkins & Stone, 1983; Hoehn & White, 1989). In the present experiments, the type of glutamate receptor involved in the indirect inhibitory effect of glutamate was examined. The selective ionotropic glutamate receptor agonists AMPA, kainate and NMDA were used, as well as the agonist at metabotropic glutamate receptors 1-amino-1,3-cyclopentanedicarboxylate (ACPD), and the antagonists 2-amino-5-phosphonopentanoate (AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (for reviews see Collingridge & Lester, 1989; Schoepp *et al.*, 1990; Watkins *et al.*, 1990). Some of these results have been reported to the Deutsche Gesellschaft für Pharmakologie und Toxikologie (von Kügelgen *et al.*, 1992b).

Methods

Rabbits of either sex and weighing 1.9–2.6 kg were killed by cervical dislocation and exsanguination. The brain was quick-

¹ Author for correspondence.

ly removed and chilled. Two slabs were cut from the occipito-parietal cortex, parallel to the surface. The superficial slab, 0.3 mm thick, consisted of pia mater, lamina I and in some cases part of lamina II; the second, deeper slab, 0.4 mm thick, consisted of laminae II–IV and in some cases parts of laminae I or V (Reichenbacher *et al.*, 1982). Unless stated otherwise, the superficial slab was discarded and round slices, 3.5 mm diameter, were punched from the second slab. Six to eight slices were preincubated at 37°C for 30 min in 2 ml medium containing (–)-[³H]-noradrenaline 0.1 µM. In some experiments, 3.5 mm diameter slices were also prepared from the superficial slab, and slices from occipital and parietal areas and from the superficial and the deeper layer were incubated with [³H]-noradrenaline separately. Following the labelling incubation, the slices were washed three times with 3 ml [³H]-noradrenaline-free medium. One slice was 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 [³H]-noradrenaline-free medium for 116 min at a rate of 1 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 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 (S₁) and 98 (S₂) min of superfusion; each consisted of a train of 6 pulses at 100 Hz. The collection of successive 3 min superfusate samples began after 50 min of superfusion, i.e., 9 min before S₁. Glutamate receptor agonists or their solvent (water) were added to the medium between S₁ and S₂ (24 min before S₂ unless stated otherwise) and kept for the remainder of the experiment; the delay from addition to arrival at tissue was about 60 s. Other drugs were added to the medium throughout superfusion. 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.

Unless stated otherwise, the medium used for incubation and superfusion contained (mM): NaCl 118, KCl 4.8, CaCl₂ 1.3, MgSO₄ 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 11, ascorbic acid 0.57, disodium EDTA 0.03. It was saturated with 5% CO₂ in O₂. 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 uptake₁. In some experiments, incubation and superfusion media were Mg²⁺-free.

The outflow of tritium was expressed as fractional rate (min⁻¹), i.e., (tritium outflow per 3 min)/[3 × (tritium present in the slice at the onset of the respective collection period)]. The electrically evoked overflow of tritium was calculated as the difference between the total overflow (during the 12 min after electrical stimulation) and the estimated basal outflow; the basal outflow was assumed to decline linearly from the 3 min interval before, to the interval 12–15 min after, electrical stimulation. The transient overflow of tritium evoked by glutamate receptor agonists themselves, when added between S₁ and S₂, was calculated in an analogous manner: as the difference between the total overflow (from addition to the 3 min interval before S₂) and the estimated basal outflow; the basal outflow was assumed to decline linearly from the 3 min interval before addition to the 3 min interval before S₂. The differences (total minus basal; Bq) were expressed as a percentage of the tritium content (Bq) of the slice at the onset of electrical stimulation or glutamate receptor agonist addition, respectively. For further evaluation of basal tritium efflux, ratios were calculated of the fractional rate of outflow in the 3 min interval before S₂ and the 3 min interval before S₁ (b₂/b₁). For further evaluation of the electrically evoked overflow, ratios were calculated of the overflow elicited by S₂

and the overflow elicited by S₁ (S₂/S₁). S₂/S₁ ratios obtained in individual experiments with a glutamate receptor agonist were calculated as a percentage of the average S₂/S₁ ratio in the appropriate control group.

Materials

The following drugs were used: (–)-[ring-2,5,6-³H]-noradrenaline, specific activity 1.94 or 2.11 TBq mmol⁻¹ (Du Pont, Dreieich, Germany), (±)-*cis*-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD; IUPAC nomenclature; see the Editorial in Schoepp *et al.*, 1990), (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid HBr (AMPA), 6-cyano-7-nitroquinoline-2,3-dione (CNQX), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 2-hydroxysaclofen (Research Biochemicals, Natick, MA, U.S.A.), yohimbine HCl (Roth, Karlsruhe, Germany), adenosine deaminase type VII from calf intestinal mucosa (EC 3.5.4.4), (±)-2-amino-5-phosphonopentanoic acid (AP5), (–)-bicuculline methiodide, desipramine HCl, kainic acid, N-methyl-D-aspartic acid (NMDA), N^G-nitro-L-arginine methyl ester HCl (L-NAME) (Sigma, Deisenhofen, Germany). Solutions of drugs were prepared with either distilled water or (2-hydroxysaclofen) the KH₂PO₄- and NaHCO₃-containing stock solution of the medium or (CNQX and DPCPX) dimethyl sulphoxide (Roth, Karlsruhe, Germany, final concentration about 0.4 mM). Dimethyl sulphoxide was the solvent for CNQX and DPCPX only, but was added in all experiments throughout superfusion to make them directly comparable.

Figure 1 Time course of tritium outflow from rabbit brain cortex slices and effects of α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA; a), kainate (b) and N-methyl-D-aspartate (NMDA; c and d). After preincubation with [³H]-noradrenaline, slices were superfused and stimulated twice (S₁ and S₂; 6 pulses/100 Hz). In some experiments, incubation and superfusion media were Mg²⁺-free (d) instead of containing the normal 1.2 mM Mg²⁺ (a–c). Glutamate receptor agonists were added as indicated. Mean ± s.e.mean of 9 (a), 21 (b), 14 (c) and 6 (d) experiments. Abscissae, min of superfusion.

Table 1 Basal tritium outflow (b_1), electrically evoked tritium overflow (S_1), and effects of drugs

Drugs added throughout superfusion	b_1 (min^{-1})	S_1 (% of tissue tritium)	n
<i>Magnesium 1.2 mM</i>			
–	0.00173 ± 0.00003^a	0.760 ± 0.020^b	195
DPCPX 10 nM	$0.00200 \pm 0.00004^{**}$	$1.252 \pm 0.032^{**}$	108
Adenosine deaminase 3 u ml^{-1}	$0.00240 \pm 0.00008^{**}$	$1.480 \pm 0.077^{**}$	18
Yohimbine $1 \mu\text{M}$	0.00182 ± 0.00009	$1.024 \pm 0.071^{**}$	30
Bicuculline $50 \mu\text{M}$	$0.00135 \pm 0.00004^{**}$	0.898 ± 0.044	18
2-Hydroxysaclofen $50 \mu\text{M}$	$0.00143 \pm 0.00005^*$	0.847 ± 0.050	17
L-NAME $100 \mu\text{M}$	$0.00135 \pm 0.00005^{**}$	$1.021 \pm 0.078^*$	20
AP5 $100 \mu\text{M}$	0.00153 ± 0.00005	0.864 ± 0.056	24
CNQX $30 \mu\text{M}$	0.00203 ± 0.00023	$0.938 \pm 0.059^*$	35
CNQX $100 \mu\text{M}$	0.00154 ± 0.00007	$1.416 \pm 0.068^{**}$	18
<i>Magnesium absent</i>			
–	0.00176 ± 0.00003	1.035 ± 0.027	77
DPCPX 10 nM	$0.00198 \pm 0.00006^{**}$	$1.450 \pm 0.051^{**}$	29

After preincubation with [^3H]-noradrenaline, cortical slices were superfused with medium containing the drugs indicated (and in addition desipramine $1 \mu\text{M}$ which is not noted since it was always present throughout superfusion). S_1 was applied after 59 min of superfusion and consisted of 6 pulses/100 Hz. b_1 refers to the 3-min period immediately before S_1 . AP5, 2-amino-5-phosphonopentanoate; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; L-NAME, N^G -nitro-L-arginine methyl ester. Means $\pm$ s.e.mean of n experiments.

^aCorresponding to $14.0 \pm 0.3 \text{ Bq min}^{-1}$.

^bCorresponding to $61.6 \pm 1.9 \text{ Bq}$.

Significant differences from corresponding experiments without the drug: $^*P < 0.05$ and $^{**}P < 0.01$.

Figure 2 Glutamate receptor agonist-induced overflow of tritium (a) and inhibition of electrically evoked tritium overflow (b). Slices were stimulated twice (S_1 and S_2). α -Amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA, $\blacksquare$), kainate ($\blacklozenge$) or N-methyl-D-aspartate (NMDA, $\blacktriangle$ and $\bullet$) was added 24 min before S_2 . In some experiments with NMDA, incubation and superfusion media were Mg^{2+} -free ($\bullet\cdots\bullet$). Abscissae, concentration of glutamate receptor agonists. For evaluation of electrically evoked tritium overflow, S_2/S_1 ratios obtained in presence of agonists were expressed as a percentage of the average corresponding control S_2/S_1 ratio. Means $\pm$ s.e.mean of 4–21 experiments. Significant differences from control: $^*P < 0.05$ and $^{**}P < 0.01$.

Statistics

Means $\pm$ s.e.mean are given throughout. Differences between means were tested for significance by the Mann-Whitney test. $P < 0.05$ or lower was the significance criterion. 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 six pulses delivered at 100 Hz markedly increased the outflow of tritium (Figure 1). Values for the magnitude of the basal outflow of tritium before S_1 and for the overflow elicited by S_1 are summarized in Table 1. In the absence of drugs (except desipramine $1 \mu\text{M}$ which was in the superfusion medium in all experiments; magnesium present), the fractional rate of tritium efflux immediately before S_1 averaged 0.00173 min^{-1} , and stimulation by six pulses/100 Hz caused an average overflow (S_1) of 0.760% of the tritium content of the tissue. Drug-induced changes will be mentioned in the appropriate sections below.

In control experiments in which the medium was drug-free (except for desipramine; magnesium present) and solvent (water) was added before S_2 , the b_2/b_1 ratio was 0.87 ± 0.01 and the S_2/S_1 ratio 1.06 ± 0.01 ($n = 33$). The average b_2/b_1 ratio also was slightly below unity, and the S_2/S_1 ratio close to unity, when solvent was added before S_2 in any other group defined in Table 1 (not shown).

Effects of glutamate receptor agonists

When added 24 min before S_2 at a concentration of $100 \mu\text{M}$, each of the three agonists after which the ionotropic glutamate receptors are named – AMPA, kainate and NMDA – elicited two effects (Figure 1). First, there was an immediate steep rise in tritium outflow. The increase was transient, and the efflux of tritium returned to baseline well before S_2 so that b_2/b_1 ratios did not differ from the corresponding control (solvent before S_2). This will be called the immediate releasing effect. The second effect was a decrease of the overflow elicited by electrical stimulation (S_2). In the case of NMDA, both effects were obtained in the presence (Figure 1c) as well as in the absence of Mg^{2+} (Figure 1d).

Concentration-response curves are shown in Figure 2. As can be seen from the error bars, the immediate releasing effect (Figure 2a) was very variable, more variable than the inhibition of the electrically evoked overflow of tritium (Figure 2b). Concentration-response curves for the inhibitory effect of kainate, and of NMDA in the presence of Mg^{2+} , were bell-shaped (Figure 2b). Kainate, and NMDA in the absence of Mg^{2+} , were more potent in inhibiting the electrically evoked overflow than in eliciting the immediate release: marked inhibition was obtained at $10 \mu M$, a concentration at which there was not even a tendency for the releasing effect. AMPA, and NMDA in the presence of Mg^{2+} , produced release and inhibition of electrically evoked overflow at the same concentrations. AMPA was the most potent agonist with respect to either effect.

The question was studied whether the variability of the immediate releasing effect of NMDA (Figure 2) was due to the use of slices from different parts of the occipito-parietal cortex. Slices from the occipital area on the one hand, and the parietal area on the other, were studied separately. Moreover, slices from the outer 0.3 mm cortical layer which normally was discarded were examined in comparison with

the standard slices from 0.3–0.7 mm below the surface. No difference was found: NMDA $100 \mu M$, in Mg^{2+} -free medium, elicited increases in tritium outflow similar in magnitude and variability, irrespective of the area and layer chosen ($n = 6-8$ for each group; not shown).

In some experiments carried out in the presence of Mg^{2+} , NMDA $100 \mu M$ was added 3 or 12 min instead of the usual 24 min before S_2 . The electrically evoked overflow of tritium was evaluated only if the immediate releasing effect of NMDA was so small that b_2/b_1 in the individual slice was below 1.1 (and b_2/b_1 ratios on average did not differ significantly from control). The overflow of tritium evoked by S_2 was significantly ($P < 0.01$) reduced to $59.9 \pm 6.8\%$ of control after 3 min ($n = 5$) and to $56.7 \pm 4.7\%$ after 12 min of contact with NMDA ($n = 5$), values not significantly different from the $71.7 \pm 4.7\%$ (Figure 2b) obtained after 24 min of contact with NMDA $100 \mu M$ ($n = 14$).

The selective agonist at metabotropic glutamate receptors, ACPD, when added 24 min before S_2 at concentrations of 10 and $100 \mu M$, caused no immediate release and did not change the b_2/b_1 ratio. The electrically evoked overflow of tritium (S_2) also remained unchanged ($n = 3-5$).

Possible mediators

In a search for possible mediators of the inhibitory effects of AMPA, kainate and NMDA, their interaction with other compounds was examined that were added throughout superfusion. The interaction with the adenosine A_1 -receptor antagonist, DPCPX (Bruns *et al.*, 1987; Lohse *et al.*, 1987) was studied most extensively. DPCPX greatly attenuated or abol-

Figure 3 Interaction of glutamate receptor agonists with 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) on electrically evoked tritium overflow. Slices were stimulated twice (S_1 and S_2). α -Amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA; a), kainate (b) or N-methyl-D-aspartate (NMDA; c and d) was added 24 min before S_2 . In some experiments, incubation and superfusion media were Mg^{2+} -free (d) instead of containing the normal $1.2 \text{ mM } Mg^{2+}$ (a-c). Filled symbols represent experiments in which the medium was DPCPX-free (from Figure 2). Open symbols represent experiments in which the medium contained DPCPX 10 nM throughout superfusion. Abscissae, concentration of glutamate receptor agonists. For evaluation of electrically evoked tritium overflow, S_2/S_1 ratios obtained in presence of agonists were expressed as a percentage of the average corresponding control S_2/S_1 ratio. Means $\pm$ s.e. mean of 4–21 experiments. Significant differences from control (no agonist): * $P < 0.05$ and ** $P < 0.01$. Significant differences from experiments without DPCPX: # $P < 0.05$ and ## $P < 0.01$.

Figure 4 Interaction of glutamate receptor agonists with adenosine deaminase (ADA), yohimbine, bicuculline (Bicuc), 2-hydroxysaclofen (OH-Saclofen) and N^G -nitro-L-arginine methyl ester (L-NAME) on electrically evoked tritium overflow. Slices were stimulated twice (S_1 and S_2). α -Amino-3-hydroxy-5-methylisoxazole-4-propionate $100 \mu M$ (A; horizontally striped columns), kainate $100 \mu M$ (K; crosshatched columns), N-methyl-D-aspartate $100 \mu M$ (N; solid columns) or solvent (C, control; open columns) was added 24 min before S_2 . Other drugs were present throughout superfusion; experiments without these interacting drugs are from Figure 2. For evaluation of electrically evoked tritium overflow, S_2/S_1 ratios obtained in presence of agonists were expressed as a percentage of the average corresponding control S_2/S_1 ratio. Means $\pm$ s.e. mean of 4–33 experiments. Significant differences from C, * $P < 0.05$ and ** $P < 0.01$. Significant differences from experiments without interacting drugs: # $P < 0.05$ and ## $P < 0.01$.

ished the depression, by a wide range of concentrations of any of the three agonists, of the electrically evoked overflow of tritium (Figure 3). The inhibition by AMPA, kainate and NMDA 100 μ M was also abolished by adenosine deaminase (Figure 4). In contrast, no attenuation of the inhibition was observed in the presence of the α_2 -adrenoceptor antagonist yohimbine, the γ -aminobutyric acid_A (GABA_A) receptor antagonist bicuculline, the GABA_B receptor antagonist 2-hydroxysaclofen (Kerr *et al.*, 1988) and the inhibitor of NO synthase, N^G-nitro-L-arginine methyl ester (L-NAME; Rees *et al.*, 1990) (Figure 4). The immediate releasing effects of AMPA, kainate and NMDA were not changed by any of the interacting drugs, except for a significant ($P < 0.05$) increase of the effect of kainate 100 μ M by L-NAME and a marked but statistically non-significant reduction of the effect of NMDA 100 μ M by DPCPX (not shown).

The interacting drugs caused changes in b_1 and S_1 values (Table 1). For example, DPCPX, adenosine deaminase, yohimbine and L-NAME increased the evoked overflow of tritium (S_1).

Receptors involved

The receptors involved in the inhibitory effects of AMPA, kainate and NMDA were further studied by means of the antagonists AP5, which is selective for NMDA receptors (Davies *et al.*, 1981; Evans *et al.*, 1982), and CNQX, which selectively blocks non-NMDA receptors (Fletcher *et al.*, 1988; Honoré *et al.*, 1988). The agonists were tested at a concentration of 100 μ M (Figure 5). AP5 100 μ M abolished the effect of NMDA but failed to alter the effects of AMPA and kainate. CNQX 30 and 100 μ M, on the other hand, significantly attenuated the inhibition produced by AMPA

Figure 5 Interaction of glutamate receptor agonists with 2-amino-5-phosphopentanoate (AP5) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) on electrically evoked tritium overflow. Slices were stimulated twice (S_1 and S_2). α -Amino-3-hydroxy-5-methylisoxazole-4-propionate 100 μ M (A; horizontally striped columns), kainate 100 μ M (K; crosshatched columns), N-methyl-D-aspartate 100 μ M (N; solid columns) or solvent (C, control; open columns) was added 24 min before S_2 . AP5 and CNQX were present throughout superfusion; experiments without AP5 and CNQX are from Figure 2. For evaluation of electrically evoked tritium overflow, S_2/S_1 ratios obtained in presence of agonists were expressed as a percentage of the average corresponding control S_2/S_1 ratio. Means $\pm$ s.e. mean of 4–33 experiments. Significant differences from C, * $P < 0.05$ and ** $P < 0.01$. Significant differences from experiments without AP5 and CNQX: ## $P < 0.01$.

but did not change the inhibition produced by NMDA and kainate (Figure 5). The same pattern was found for the immediate releasing effects of the agonists: AP5 100 μ M selectively abolished the effect of NMDA, whereas CNQX 30 and 100 μ M selectively abolished the effect of AMPA (not shown).

The only change in b_1 and S_1 values was an increase by CNQX of the electrically evoked overflow of tritium (S_1 ; Table 1).

Discussion

Under the conditions used, the electrically evoked overflow of tritium represents an action potential-induced, neuronal release of [3 H]-noradrenaline (see Reichenbacher *et al.*, 1982). Similarly, the immediate increase in tritium outflow elicited by AMPA, kainate and NMDA certainly reflected a glutamate receptor-mediated release of [3 H]-noradrenaline (see Introduction). Brief high-frequency pulse trains were used for electric stimulation in order to minimize the presynaptic α_2 -autoinhibition of the release of [3 H]-noradrenaline (Singer, 1988) which may cloud effects mediated by other presynaptic receptors (Allgaier *et al.*, 1987; Limberger *et al.*, 1988). Yohimbine nevertheless increased the release of [3 H]-noradrenaline elicited by S_1 (Table 1). However, the increase was slight. Moreover, differences between S_1 (and b_1) values must be viewed with caution since this kind of study is planned to be compared S_2/S_1 (and b_2/b_1) ratios; conclusions from minor changes in S_1 (b_1) should be drawn only when there is additional evidence; the increase caused by DPCPX and adenosine deaminase, for example (Table 1), is a real change, also obtained when these compounds are added before S_2 and reflecting removal of an inhibition by endogenous adenosine (von Kugelgen *et al.*, 1992a,c). Desipramine was present throughout superfusion in all experiments for two reasons: to increase the signal measured, i.e. electrically evoked overflow of tritium, by inhibition of [3 H]-noradrenaline re-uptake; and to rule out the possibility that drug effects were due to changes in this re-uptake.

AMPA, kainate and NMDA all reduced the electrically evoked release of [3 H]-noradrenaline. Apart from this basic fact which confirms and extends previous findings with glutamate (von Kugelgen *et al.*, 1992a), the present study allows us to draw two main conclusions: that, as in the case of glutamate, the inhibition was due to adenosine; and that it can be elicited by activation of each of the three types of ionotropic glutamate receptor, AMPA, kainate and NMDA receptors, but not by activation of metabotropic glutamate receptors.

In support of the first conclusion, both the A_1 antagonist DPCPX and adenosine deaminase attenuated and in most cases even abolished the inhibitory effects of AMPA, kainate and NMDA. The blockade occurred without simultaneous suppression of the immediate releasing effect of AMPA, kainate and NMDA and, hence, was not due to some antagonism at glutamate receptors. The interaction is exactly analogous to the interaction of DPCPX and adenosine deaminase with glutamate (von Kugelgen *et al.*, 1992a), indicating that, as far as the role of adenosine is concerned, the mechanisms of action are identical. None of the release-inhibiting effects was attenuated by yohimbine, bicuculline, 2-hydroxysaclofen or L-NAME, indicating that neither an activation of presynaptic α_2 -adrenoceptors nor release of GABA or an induction of NO synthesis was involved.

The involvement of AMPA receptors in the effect of AMPA, of kainate receptors in the effects of kainate, and of NMDA receptors in the effect of NMDA is supported by the fact that the agonists produced inhibition at concentrations (10–30 μ M) at which they elicit other effects through these respective receptors, most interestingly perhaps the immediate release of noradrenaline (Jones *et al.*, 1987; Hoehn *et al.*, 1990; Fink *et al.*, 1989; 1992; Pittaluga & Raiteri, 1992; and see below). Moreover, only the inhibition of NMDA but not

the inhibition by AMPA and kainate was blocked by the selective NMDA receptor antagonist, AP5. Conversely, only the inhibition by AMPA but not the inhibition by NMDA and kainate was significantly attenuated by CNQX, the ionotropic glutamate receptor affinity of which declines in the order AMPA > kainate >> NMDA (Fletcher *et al.*, 1988; Honoré *et al.*, 1988). Due to lack of an appropriate antagonist, selective blockade of the effect of kainate could not be demonstrated. However, the fact that CNQX attenuated the effect of AMPA but not of kainate, although the concentration of AMPA and kainate chosen (100 μ M) occupied a similar position on each agonist's concentration-response curve (Figure 2b), is best explained by an action of kainate at a separate, i.e. the kainate, receptor.

Our receptor characterization refers directly to the glutamate receptors involved in inhibition of [3 H]-noradrenaline release, but indirectly it also shows that AMPA, kainate and NMDA receptors, when activated, all elicit release of adenosine (or an adenosine nucleotide which is then degraded to adenosine). In agreement with this conclusion from rabbit brain slices it has been demonstrated directly that activation of any of the three ionotropic glutamate receptor releases adenosine in rat brain cortex slices (Hoehn & White, 1990; Craig & White, 1993). There are additional parallels. The release by NMDA of adenosine from rat brain cortex slices, like the inhibition by NMDA of the release of [3 H]-noradrenaline from rabbit brain cortex slices, was observed both in the absence and in the presence of Mg^{2+} (Hoehn *et al.*, 1990, and Figure 2b). In rat brain cortex slices in the absence of Mg^{2+} , NMDA was more potent in releasing adenosine than [3 H]-noradrenaline (Hoehn *et al.*, 1990), and similarly in rabbit brain cortex slices in the absence of Mg^{2+} , NMDA was more potent in inhibiting electrically evoked [3 H]-noradrenaline release than in releasing [3 H]-noradrenaline (Figure 2). The possibility of a release of adenosine following activation of metabotropic glutamate receptors has not been studied previously; the lack of effect of ACPD in our experiments suggests that such release does not occur.

For reasons we still do not understand, glutamate itself failed to release [3 H]-noradrenaline from rabbit cortical slices

(von Kügelgen *et al.*, 1992a). AMPA, kainate and NMDA, on the other hand, all caused an immediate transient release in the rabbit. In terms of antagonism, the receptors involved behaved like those mediating the inhibition of electrically evoked release, indicating that, as in other species (see Introduction), all three ionotropic glutamate receptors can elicit the immediate releasing effect. The noradrenaline releasing effect of NMDA was blocked by Mg^{2+} in human, rat and mouse brain slices (Vezzani *et al.*, 1987; Fink *et al.*, 1989; 1992; Hoehn *et al.*, 1990; Zhao *et al.*, 1990) but not in rabbit brain slices (Figure 2a), possibly because the rabbit noradrenergic terminal axons were slightly depolarized under our conditions. It should be noted that the immediate releasing effect was not responsible for the subsequent inhibition of electrically evoked release: in the case of kainate, and of NMDA in the absence of Mg^{2+} , the inhibition occurred at concentrations below the [3 H]-noradrenaline-releasing threshold, and DPCPX as well as adenosine deaminase abolished the inhibition but not the releasing effect. The metabotropic receptor agonist, ACPD, once again did not share the effect of the ionotropic receptor agonists.

Glutamate, acting on NMDA receptors, and adenosine inhibited the same ω -conotoxin-sensitive, dihydropyridine-resistant, N-type Ca^{2+} current in rat hippocampal neurones (Chernesvkaya *et al.*, 1991). One explanation, not discussed by the authors, would be that the effect of glutamate was mediated by adenosine. Inhibition of N-type Ca^{2+} channels is a possible mechanism triggered by activation of prejunctional A_1 -purinoceptors (see Fredholm & Hu, 1993). The Ca^{2+} channel effect (Chernesvkaya *et al.*, 1991) and the release-inhibiting effect (present study), hence, may be serial events following the activation of ionotropic EAA receptors, release of adenosine and activation by adenosine of prejunctional A_1 -receptors.

We thank Dr R.B. Illing (Unit for Morphological Brain Research, HNO-Klinik, Freiburg, Germany) for histological analysis of the brain slices. The study was supported by the Jung-Stiftung, Hamburg, and the Deutsche Forschungsgemeinschaft (SFB 325).

References

- ALLGAIER, C., HERTTING, G. & VON KÜGELGEN, O. (1987). The adenosine receptor-mediated inhibition of noradrenaline release possibly involves a N-protein and is increased by α_2 -autoreceptor blockade. *Br. J. Pharmacol.*, **90**, 403–412.
- BLANDINA, P., JOHNSON, D., WALCOTT, J. & GOLDFARB, J. (1992). Release of endogenous norepinephrine from rat hypothalamus by stimulation of N-methyl-D-aspartic acid receptors. *J. Pharmacol. Exp. Ther.*, **263**, 61–68.
- 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.
- CHERNEVSKAYA, N.I., OBUKHOV, A.G. & KRISHTAL, O.A. (1991). NMDA receptor agonists selectively block N-type calcium channels in hippocampal neurons. *Nature*, **349**, 418–420.
- COLLINGRIDGE, G.L. & LESTER, R.A.J. (1989). Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol. Rev.*, **40**, 143–210.
- CRAIG, C.G. & WHITE, T.D. (1993). N-Methyl-D-aspartate- and non-N-methyl-D-aspartate-evoked adenosine release from rat cortical slices: distinct purinergic sources and mechanisms of release. *J. Neurochem.*, **60**, 1073–1080.
- DAVIES, J., FRANCIS, A.A., JONES, A.W. & WATKINS, J.C. (1981). 2-Amino-5-phosphonovaleate (2APV), a potent and selective antagonist of amino acid-induced and synaptic excitation. *Neurosci. Lett.*, **21**, 77–81.
- EVANS, R.H., FRANCIS, A.A., JONES, A.W., SMITH, D.A.S. & WATKINS, J.C. (1982). The effects of a series of ω -phosphonic α -carboxylic amino acids on electrically evoked and excitant amino acid-induced responses in isolated spinal cord preparations. *Br. J. Pharmacol.*, **75**, 65–75.
- FINK, K. & GÖTHERT, M. (1992). Presynaptic site of action underlying the ethanol-induced inhibition of norepinephrine release evoked by stimulation of N-methyl-D-aspartate (NMDA) receptors in rat cerebral cortex. *Brain Res.*, **572**, 27–32.
- FINK, K., GÖTHERT, M., MOLDERINGS, G. & SCHLICKER, E. (1989). N-Methyl-D-aspartate (NMDA) receptor-mediated stimulation of noradrenaline release, but not release of other neurotransmitters, in the rat brain cortex: receptor location, characterization and desensitization. *Naunyn-Schmied. Arch. Pharmacol.*, **339**, 514–521.
- FINK, K., SCHULTHEISS, R. & GÖTHERT, M. (1992). Stimulation of noradrenaline release in human cerebral cortex mediated by N-methyl-D-aspartate (NMDA) and non-NMDA receptors. *Br. J. Pharmacol.*, **106**, 67–72.
- FLETCHER, E.J., MARTIN, D., ARAM, J.A., LODGE, D. & HONORÉ, T. (1988). Quinoxalinediones selectively block quisqualate and kainate receptors and synaptic events in rat neocortex and hippocampus and frog spinal cord *in vitro*. *Br. J. Pharmacol.*, **95**, 585–597.
- FREDHOLM, B.B. & HU, P.S. (1993). Effect of an intracellular calcium chelator on the regulation of electrically evoked [3 H]-noradrenaline release from rat hippocampal slices. *Br. J. Pharmacol.*, **108**, 126–131.
- HOEHN, K., CRAIG, C.G. & WHITE, T.D. (1990). A comparison of N-methyl-D-aspartate-evoked release of adenosine and [3 H]-norepinephrine from rat cortical slices. *J. Pharmacol. Exp. Ther.*, **255**, 174–181.
- HOEHN, K. & WHITE, T.D. (1989). Evoked release of endogenous adenosine from rat cortical slices by K^+ and glutamate. *Brain Res.*, **478**, 149–151.

- HOEHN, K. & WHITE, T.D. (1990). N-Methyl-D-aspartate, kainate and quisqualate release endogenous adenosine from rat cortical slices. *Neuroscience*, **39**, 441–450.
- HONORÉ, T., DAVIES, S.N., DREJER, J., FLETCHER, E.J., JACOBSEN, P., LODGE, D. & NIELSEN, F.E. (1988). Quinoxalinediones: potent competitive non-NMDA glutamate receptor antagonists. *Science*, **241**, 701–703.
- JHAMANDAS, K. & DUMBRILLE, A. (1980). Regional release of [³H]adenosine derivatives from rat brain in vivo: effect of excitatory amino acids, opiate agonists, and benzodiazepines. *Can. J. Physiol. Pharmacol.*, **58**, 1262–1278.
- JONES, S.M., SNELL, L.D. & JOHNSON, K.M. (1987). Phencyclidine selectively inhibits N-methyl-D-aspartate-induced hippocampal [³H]norepinephrine release. *J. Pharmacol. Exp. Ther.*, **240**, 492–497.
- KERR, D.I.B., ONG, J., JOHNSTON, G.A.R., ABBENANTE, J. & PRAGER, R.H. (1988). 2-Hydroxy-saclofen: an improved antagonist at central and peripheral GABA_B receptors. *Neurosci. Lett.*, **92**, 92–96.
- LIMBERGER, N., SPÄTH, L. & STARKE, K. (1988). Presynaptic α_2 -adrenoceptor, opioid κ -receptor and adenosine A₁-receptor interactions on noradrenaline release in rabbit brain cortex. *Naunyn-Schmied. Arch. Pharmacol.*, **338**, 53–61.
- 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 A₁ adenosine receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **336**, 204–210.
- NELSON, M.F., ZACZEK, R. & COYLE, J.T. (1980). Effects of sustained seizures produced by intrahippocampal injection of kainic acid on noradrenergic neurons: evidence for local control of norepinephrine release. *J. Pharmacol. Exp. Ther.*, **214**, 694–702.
- PERKINS, M.N. & STONE, T.W. (1983). In vivo release of [³H]-purines by quinolinic acid and related compounds. *Br. J. Pharmacol.*, **80**, 263–267.
- PITTALUGA, A. & RAITERI, M. (1992). N-Methyl-D-aspartic acid (NMDA) and non-NMDA receptors regulating hippocampal norepinephrine release. I. Location on axon terminals and pharmacological characterization. *J. Pharmacol. Exp. Ther.*, **260**, 232–237.
- PULL, I. & MCILLWAIN, H. (1975). Actions of neurohumoral agents and cerebral metabolites on output of adenine derivatives from superfused tissues of the brain. *J. Neurochem.*, **24**, 695–700.
- REES, D.D., PALMER, R.M.J., SCHULZ, R., HODSON, H.F. & MONCADA, S. (1990). Characterization of three inhibitors of endothelial nitric oxide synthase *in vitro* and *in vivo*. *Br. J. Pharmacol.*, **101**, 746–752.
- REICHENBACHER, D., REIMANN, W. & STARKE, K. (1982). α -Adrenoceptor-mediated inhibition of noradrenaline release in rabbit brain cortex slices. *Naunyn-Schmied. Arch. Pharmacol.*, **319**, 71–77.
- SCHOEPP, D., BOCKAERT, J. & SLADCEK, F. (1990). Pharmacological and functional characteristics of metabotropic excitatory amino acid receptors. *Trends Pharmacol. Sci.*, **11**, 508–515.
- SINGER, E.A. (1988). Transmitter release from brain slices elicited by single pulses: a powerful method to study presynaptic mechanisms. *Trends Pharmacol. Sci.*, **9**, 274–276.
- VEZZANI, A., WU, H.Q. & SAMANIN, R. (1987). [³H]Norepinephrine release from hippocampal slices is an *in vitro* biochemical tool for investigating the pharmacological properties of excitatory amino acid receptors. *J. Neurochem.*, **49**, 1438–1442.
- VON KÜGELGEN, I., SPÄTH, L. & STARKE, K. (1992a). Adenosine but not an adenine nucleotide mediates tonic purinergic inhibition, as well as inhibition by glutamate, of noradrenaline release in rabbit brain cortex slices. *Naunyn-Schmied. Arch. Pharmacol.*, **346**, 677–684.
- VON KÜGELGEN, I., SPÄTH, L. & STARKE, K. (1992b). A purinergic mechanism is involved in the inhibitory effects of glutamate, N-methyl-D-aspartate and kainic acid but not of methoxamine on the electrically evoked release of noradrenaline in rabbit brain cortex. *Naunyn-Schmied. Arch. Pharmacol.*, **346**, R60.
- VON KÜGELGEN, I., SPÄTH, L. & STARKE, K. (1992c). Stable adenine nucleotides inhibit [³H]-noradrenaline release in rabbit brain cortex slices by direct action at presynaptic adenosine A₁-receptors. *Naunyn-Schmied. Arch. Pharmacol.*, **346**, 187–196.
- WANG, J.K.T., ANDREWS, H. & THUKRAL, V. (1992). Presynaptic glutamate receptors regulate noradrenaline release from isolated nerve terminals. *J. Neurochem.*, **58**, 204–211.
- WATKINS, J.C., KROGSGAARD-LARSEN, P. & HONORÉ, T. (1990). Structure-activity relationships in the development of excitatory amino acid receptor agonists and competitive antagonists. *Trends Pharmacol. Sci.*, **11**, 25–33.
- ZHAO, X.H., KITAMURA, Y. & NOMURA, Y. (1990). Involvement of glutamate receptor subtypes in L-[³H]noradrenaline release from cerebral cortical and hippocampal slices of mice. *Neurochem. Int.*, **16**, 11–16.

(Received June 18, 1993)

Revised August 11, 1993

Accepted August 13, 1993)