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# The adenosine receptor-mediated inhibition of noradrenaline release possibly involves a N-protein and is increased by $\alpha_2$-autoreceptor blockade

Clemens Allgaier[1], Georg Hertting & Oda V. Kügelgen

Institute of Pharmacology, University of Freiburg, Hermann-Herder-Str. 5, D-7800 Freiburg, Federal Republic of Germany

**1** The stimulation-evoked overflow of [$^3$H]-noradrenaline from slices of the rabbit hippocampus is inhibited by $\alpha_2$-autoreceptors as well as by adenosine ($A_1$)-receptors. Slices of rabbit hippocampus were labelled with [$^3$H]-noradrenaline, superfused continuously and stimulated twice electrically (rectangular pulses; 2 ms, 3 Hz, 24 mA, 5 V cm$^{-1}$).

**2** Treatment of hippocampal slices with N-ethylmaleimide (NEM, 30 $\mu$M; 30 min), which functionally disturbs certain N-proteins, decreased the inhibitory action of adenosine receptor agonists like (−)-N$^6$-(R-phenylisopropyl)-adenosine ((−)-PIA) and adenosine on noradrenaline release. Release inhibition caused by (−)-PIA (0.03–1 $\mu$M) was antagonized by NEM in a non-competitive manner in the absence and in the presence of the $\alpha_2$-adrenoceptor antagonist yohimbine.

**3** The adenosine receptor antagonist 8-phenyltheophylline significantly increased the evoked noradrenaline release by about 15% in control slices by diminishing the inhibitory action of endogenous adenosine. In NEM-treated slices this effect of 8-phenytheophylline was not seen. In the presence of (−)-PIA (0.1 $\mu$M), i.e. under conditions of an increased inhibitory tone, release facilitation by 8-phenyltheophylline was decreased by NEM compared to that in the respective controls.

**4** Occupation of the $A_1$-receptor with (−)-PIA prior to and during the NEM treatment did not protect the $A_1$-receptor-coupled signal transduction system from being affected by NEM.

**5** In the presence of the $\alpha_2$-adrenoceptor antagonist yohimbine, the inhibitory action of (−)-PIA was strongly increased.

**6** The above results suggest the involvement of a regulatory N-protein in the $A_1$-receptor-mediated inhibition of noradrenaline release and an interaction between the $\alpha_2$-autoreceptor and the $A_1$-receptor-coupled signal transduction system, possibly at the level of a N-protein.

## Introduction

Extracellular signals of hormones and neurotransmitters are often transduced across the plasma membrane to the intracellular effector system via regulatory guanine-nucleotide-binding (N) proteins (Cooper, 1982; Gilman, 1984; Holz *et al.*, 1986; Cockroft & Gomperts, 1986). In brain the existence of two regulatory N-proteins has been reported by Sternweis & Robishaw (1984) and Neer *et al.* (1984). One of them seems to be identical with $N_i$, which mediates receptor-coupled inhibition of adenylate cyclase, whereas the function of the other protein, termed $N_o$, is unknown. ADP-ribosylation of the $\alpha$-subunit of $N_i$ caused by islet-activating protein (pertussis toxin) diminished or even abolished receptor-coupled inhibition of adenylate cyclase (Hildebrandt *et al.*, 1983; Murayama & Ui, 1983) and decreased, like guanine nucleotides (Rodbell, 1980), the agonist affinity for inhibitory receptors by uncoupling $N_i$ from its receptor (Kurose *et al.*, 1983; Boyer *et al.*, 1984; Cote *et al.*, 1984; Nomura *et al.*, 1985). These observations are similar to those obtained with the SH-reagent N-ethylmaleimide (NEM). NEM attenuated or abolished the $\alpha$-adrenoceptor (Jakobs *et al.*, 1982), the muscarinic receptor (Harden *et al.*, 1982) and the adenosine receptor-mediated (Ukena *et al.*, 1984; Fredholm *et al.*, 1985) inhibition of adenylate cyclase and mimicked the effect of guanine nucleotides on agonist binding to these receptors (Harden *et al.*, 1982; Yeung & Green, 1983; Quennedy *et al.*, 1984; Ukena *et al.*, 1984; Fredholm *et al.*, 1985).

[1]Author for correspondence.

As in the case of $N_i$, the α-subunit of $N_o$ is ADP-ribosylated by islet-activating protein (IAP) (Sternweis & Robishaw, 1984). Recently, Asano & Ogasawara (1986) demonstrated on cerebral cortex membranes that NEM uncoupled $N_i$ as well as $N_o$ from the γ-aminobutyric $acid_B$ ($GABA_B$)-receptor by modifying the α-subunit of the N-proteins directly at the site or near to the site which is ADP-ribosylated by IAP. This result again proves NEM as a suitable tool to explore the involvement of these regulatory N-proteins in signal transduction systems.

Regulatory N-proteins also seem to be involved in the modulation of neurotransmitter release by presynaptically-located receptors. Dolphin & Prestwich (1985) postulated the involvement of $N_i$ in the $A_1$-receptor-mediated inhibition of glutamate release from the finding that IAP prevented the (−)-$N^6$-(R-phenylisopropyl adenosine ((−)-PIA)-induced inhibition of adenylate cyclase and altered the inhibition of release to facilitation. Evidence for the involvement of a regulatory N-protein in the autoinhibitory feedback mechanism of noradrenergic nerve terminals of the rabbit hippocampus has been shown by the finding that both IAP as well as NEM diminished the autoreceptor-mediated effects on noradrenaline release (Allgaier *et al.*, 1985; 1986).

Noradrenaline release from noradrenergic nerve terminals of the rabbit hippocampus is inhibited by $\alpha_2$-autoreceptors as well as by adenosine $A_1$-, κ-opioid-, and dopamine $D_2$-receptors (Jackisch *et al.*, 1984a; 1985a,b; 1986). In this paper, data from experiments with NEM are presented which suggest the involvement of a regulatory N-protein in the $A_1$-receptor-coupled signal transduction system of the rabbit hippocampus. Our results support recent findings by Fredholm & Lindgren (1986) who reported that autoinhibition of noradrenaline release from rat hippocampal slices by (−)-PIA was abolished by NEM. In addition, we present evidence for an interaction between the $\alpha_2$-autoreceptor and the $A_1$-receptor-coupled signal transduction system.

## Methods

### *Superfusion experiments*

Superfusion experiments and NEM pretreatment were performed as described in detail by Allgaier *et al.* (1986). In brief: slices (0.4 mm thick, 5–7 mg wet weight) of the middle third of the rabbit hippocampus were prepared and subsequently incubated in a modified Krebs-Henseleit medium (composition in 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, $Na_2EDTA$ 0.03; saturated with 5% $CO_2$ in $O_2$, pH 7.4) containing 0.1 μmol $l^{-1}$ [$^3$H]-noradrenaline (44.4 Ci $mmol^{-1}$) for 30 min at 37°C. After labelling with [$^3$H]-noradrenaline, the slices were rinsed, transferred to superfusion chambers, continuously superfused at a rate of 1 ml $min^{-1}$ and stimulated twice electrically for 2 min each (rectangular pulses; 2 ms, 3 Hz, 24 mA, 5 V $cm^{-1}$) after 60 ($S_1$) and 125 min ($S_2$). In all experiments cocaine 30 μM was present throughout superfusion. At the end of the superfusion, the slices were removed from the superfusion chambers and solubilized in Soluene 350 (Packard Instrument, Frankfurt, FRG) before tritium determination.

NEM treatment of the slices was in general performed before labelling with [$^3$H]-noradrenaline. Slices were preincubated for 30 min at 37°C in medium containing NEM (30 μM). NEM preincubation did not affect the uptake of tritiated noradrenaline during the subsequent incubation in medium containing 0.1 μM [$^3$H]-noradrenaline (Allgaier *et al.*, 1986). Controls were preincubated with NEM-free medium under the same conditions. Total duration of superfusion was 150 min; the electrical stimulations were applied as described above. Adenosine receptor-ligands were added to the superfusion medium for 15 min before $S_2$ onwards. NEM pretreatment allows a direct observation of the effect of NEM on the action of exogenous adenosine-receptor-ligands by regarding the ratio $S_2/S_1$ or the differences $S_2 - S_1$ as estimates of drug effects, since autoinhibition, also affected by NEM, contributes to the amounts of the evoked overflow both at $S_1$ and $S_2$.

Receptor protection experiments were performed to exclude a direct effect of NEM on the binding site of the receptor protein. In these experiments NEM treatment of the slices occurred between the first ($S_1$) and the second stimulation period ($S_2$). $S_1$ was applied at 60 min, NEM (30 μM) was administered from 80–110 min; $S_2$ was applied at 170 min and the superfusate was collected until 195 min. Corresponding control slices were superfused with NEM-free medium. (−)-PIA was present either prior to and during (from 65 min on) or following NEM treatment (from 155 min on) for the rest of the experiment. Occupation of the $A_1$-receptor by (−)-PIA prior to and during NEM treatment should prevent the active site of the receptor from being alkylated by NEM. The fractional rate of tritium outflow (5 min)$^{-1}$ was calculated as (tritium outflow per 5 min)/(tritium content in the slice at the start of the respective 5 min collection period). The electrically-evoked overflow of tritium ($S_1$ or $S_2$, expressed as a percentage of tissue tritium at the beginning of the respective stimulation period) was calculated by subtraction of the basal outflow from the total overflow of radioactivity. The basal outflow was assumed to decline linearly from the fraction 5 min before to the fraction 20–25 min after the onset of stimulation. Effects of drugs on noradrenaline release were evaluated by calculating the ratios $S_2/S_1$ or the differences $S_2 - S_1$ of the overflow evoked by the two

stimulation periods. The ratios reflect % changes of the electrically-evoked tritium overflow, whereas the differences reflect changes in the absolute amount.

*Statistics*

Results are given as arithmetic means ± s.e.mean. Differences between the means of treatments and their corresponding controls were tested with a one-way-analysis of variance. As a preliminary to this, Bartlett's test, with a minimum level of significance of 10%, was applied to refute the null hypothesis (homogeneity of variances, goodness-of-fit for normal distribution). The significance of differences between the treated groups was determined with Students $t$ test (two-tailed) using the error mean square of the analysis of variance.

*Drugs*

The following were used: (−)-[ring-2,5,6-$^3$H]-noradrenaline (NEN, Dreieich, FRG); adenosine and N-ethylmaleimide (Sigma, München, FRG); cocaine HCl and clonidine HCl (Boehringer, Ingelheim, FRG); (−)-$N^6$-(R-phenylisopropyl)-adenosine (Boehringer, Mannheim, FRG); 8-phenyltheophylline (Calbiochem, La Jolla, CA, USA); yohimbine HCl (Merck, Darmstadt, FRG).

## Results

*Effects of N-ethylmaleimide on the action of adenosine receptor agonists and antagonists on noradrenaline release*

The electrically-evoked overflow of tritium from slices of the rabbit hippocampus, labelled with [$^3$H]-noradrenaline, was about 3.5–4% of tissue tritium. NEM preincubation (30 μM, 30 min) increased the evoked tritium overflow by about 65% ($P < 0.0001$) at the first ($S_1$) as well as at the second stimulation period ($S_2$; Figure 1a). The ratios $S_2/S_1$ of both the control and the NEM-treated slices amounted to about 1. The basal outflow of tritium which was subtracted from the total overflow of radioactivity to calculate the stimulation-evoked overflow was enhanced by about 53% ($P < 0.001$; Figure 1a).

The adenosine receptor agonist, (−)-PIA, added 15 min before $S_2$ to the superfusion medium, inhibited the evoked overflow of tritium in a concentration-dependent manner (Figure 1b). The concentration-response curve for the action of (−)-PIA (0.03–1 μM) in NEM-treated slices was flattened in comparison to the curve of control slices (Figure 1b). For instance, (−)-PIA 0.3 μM reduced the evoked overflow of tritium in control slices by 41%, but in NEM-treated slices by only 16% (Table 1).

In some experiments the $\alpha_2$-adrenoceptor agonist yohimbine (0.1 μM) was present throughout superfusion. Under these conditions the evoked tritium overflow was strongly increased by about 200% (Table 1) which is due to blockade of the $\alpha_2$-autoreceptor. In the presence of yohimbine, the inhibitory action of (−)-PIA as well as its reduction by NEM were more pronounced (Figure 1c). The inhibitory action of (−)-PIA 0.3 μM on the evoked overflow of tritium, for instance, was increased from 41% in the absence of yohimbine to 53% in the presence of yohimbine (Table 1). In NEM-pretreated slices (−)-PIA 0.3 μM inhibited the electrically-evoked overflow of tritium only by 17%. As evident from these data, the remaining potency of (−)-PIA to inhibit the evoked tritium overflow in NEM-treated slices was independent of the presence of yohimbine (Figure 1b,c).

Adenosine (30 μM) inhibited the electrically-evoked tritium overflow from slices of the rabbit hippocampus by more than 40%; in NEM-treated slices the inhibitory effect of adenosine on the evoked tritium overflow was significantly reduced to 13% (Table 1). Also, on considering the changes in the absolute amounts of evoked tritium, it is clear that the inhibitory effect of adenosine receptor agonists was diminished by NEM treatment (Table 1; $S_2 - S_1$).

In the rabbit hippocampus, adenosine receptor antagonists facilitate noradrenaline release by counteracting the inhibitory action of endogenous adenosine (Jackisch *et al.*, 1985a). In our experiments, 8-phenyltheophylline, a very potent adenosine receptor antagonist (Bruns *et al.*, 1983), caused a slight but significant ($P < 0.001$) enhancement of the evoked overflow of tritium by 15% (Table 2). A functional invalidation of the signal transduction system, should lead to a diminished inhibitory action of endogenous adenosine. Therefore, the ability of 8-phenyltheophylline to enhance the evoked overflow of tritium by $A_1$-receptor blockade should be reduced by NEM. Indeed, the 8-phenyltheophylline-dependent enhancement of the evoked overflow of tritium was not significant in NEM-treated slices (Table 2). When (−)-PIA (0.1 μM) was administered throughout superfusion, the electrically-evoked overflow of tritium in the presence of (−)-PIA was about 2.5% of tissue tritium in control slices and about 5.40% of tissue tritium in NEM-treated slices, at the first and at the second stimulation period, respectively. 8-Phenyltheophylline, applied 15 min before $S_2$, enhanced the evoked overflow of tritium by about 50% (Table 3). The enhancement of the stimulation-evoked overflow of tritium caused by 8-phenyltheophylline was reduced to 16% in NEM-treated slices; this effect of NEM is also evident on consideration of the differences $S_2 - S_1$ and hence the absolute amounts of evoked tritium (Table 3).

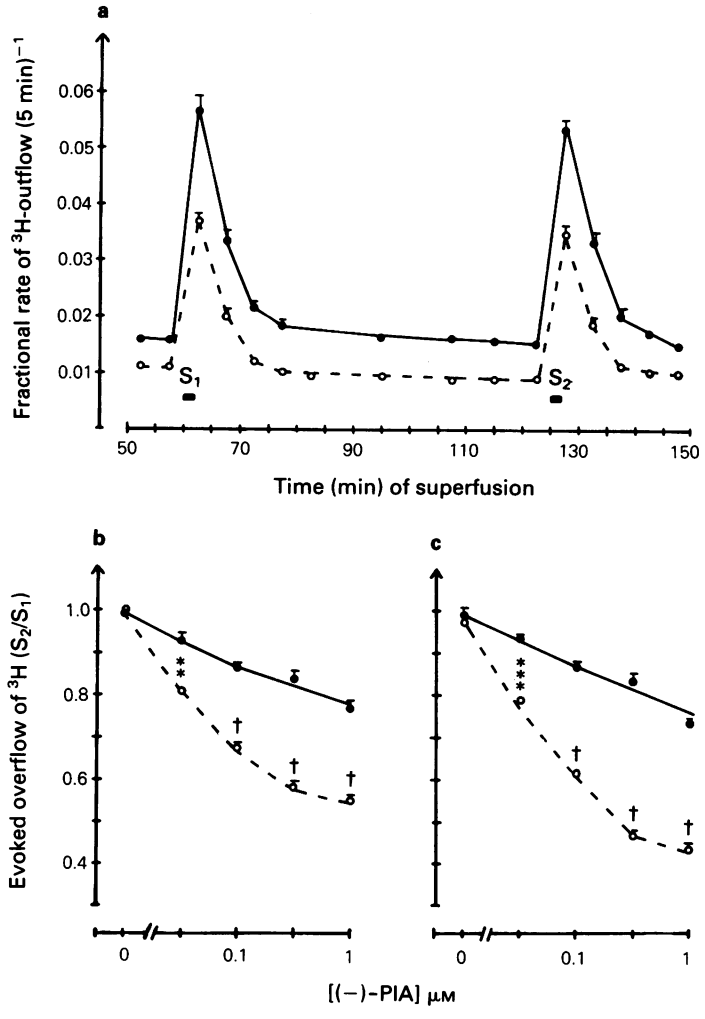


**Figure 1** Effect of N-ethylmaleimide (NEM) preincubation (a) on basal and on electrically-evoked outflow of tritium; (b) and (c) on the inhibitory action of (−)-N$^6$-(R-phenylisopropyl)-adenosine ((−)-PIA) on noradrenaline release from slices of the rabbit hippocampus incubated with [$^3$H]-noradrenaline. The slices were either preincubated in NEM-containing (30 μM; ●——●) or NEM-free medium (O---O) for 30 min, subsequently labelled with [$^3$H]-noradrenaline and then superfused continuously. During the superfusion the slices were stimulated twice electrically at 60 ($S_1$) and 125 min ($S_2$) for 2 min each (2 ms, 3 Hz, 24 mA, 5 Vcm$^{-1}$). The evoked outflow of tritium is expressed as % of tritium content at the onset of the respective stimulation period. $S_1$ was 3.70 ± 0.10% and $S_2$ was 3.69 ± 0.09% for the control slices ($n$ = 13) and 6.08 ± 0.27% ($S_1$, $P < 0.0001$ vs. controls) or 6.06 ± 0.27% ($S_2$, $P < 0.0001$ vs. controls) for the NEM preincubated slices ($n$ = 8). Fractional rates of tritium outflow are depicted as means with s.e.mean shown by vertical bars. (b) (−)-PIA (0.03–1 μM) was added 15 min before the second stimulation period. The evoked overflow of tritium of the first stimulation period ($S_1$) was 3.84 ± 0.07% ($n$ = 44) for the control slices (O---O) and 6.02 ± 0.13% ($n$ = 31; $P < 0.0001$ vs. control slices) for the NEM-treated slices (●——●). In (c), yohimbine 0.1 μM was present throughout superfusion. $S_1$ was 12.92 ± 0.57 ($n$ = 23) in control slices and 12.10 ± 0.33 ($n$ = 27) in NEM-treated slices. In (b) and (c) means of the $S_2/S_1$ ratios ± s.e.mean are given, numbers of experiments 3–8; significant differences between untreated and NEM-treated slices: $^{**}P < 0.01$; $^{***}P < 0.001$; †$P < 0.0001$.

**Table 1** Effect of N-ethylmaleimide (NEM) on the inhibitory action of adenosine receptor agonists on the electrically-evoked overflow of tritium from slices of the rabbit hippocampus

| *Drug at $S_2$ (μM)* | *Drug th. (μM)* | *NEM-treated* | *$S_1$ (%)* | *$S_2$ (%)* | *$S_2-S_1$ (%)* | n |
|---|---|---|---|---|---|---|
| (−)-PIA 0.3 | − | − | 3.94 ± 0.12 | 2.34 ± 0.80 | − 1.60 ± 0.11 | 6 |
| (−)-PIA 0.3 | − | + | 5.76 ± 0.45 | 4.84 ± 0.41 | − 0.92 ± 0.12** | 5 |
| (−)-PIA 0.3 | yohimbine 0.1 | − | 13.52 ± 0.50 | 6.36 ± 0.26 | − 7.16 ± 0.30 | 4 |
| (−)-PIA 0.3 | yohimbine 0.1 | + | 11.04 ± 0.88 | 9.13 ± 0.59 | − 1.91 ± 0.25† | 6 |
| Adenosine 30.0 | − | − | 3.68 ± 0.12 | 2.06 ± 0.12 | − 1.62 ± 0.13 | 4 |
| Adenosine 30.0 | − | + | 5.47 ± 0.17 | 4.75 ± 0.14 | − 0.72 ± 0.13** | 4 |

Slices were either preincubated in NEM-containing (30 μM) or NEM-free medium for 30 min. During the superfusion the slices were stimulated twice electrically at 60 ($S_1$) and 125 min ($S_2$). (−)-$N^6$-(R-phenylisopropyl)-adenosine ((−)-PIA, 0.03 μM) or adenosine (30 μM) were added 15 min before $S_2$ to the superfusion medium; drugs present throughout (Drug th.) superfusion, in addition to cocaine (30 μM), as indicated. $S_1$, $S_2$ and the differences $S_2-S_1$ (expressed as % of tissue tritium, respectively) of each group are given.
Means ± s.e.mean of *n* observations are given; significant differences from NEM-untreated slices: $^{**}P<0.01$; $\dagger P<0.0001$.

**Table 2** Antagonism of 8-phenyltheophylline (8-Phth) to the action of endogenous adenosine on the electrically-evoked overflow of tritium (A) in N-ethylmaleimide (NEM)-treated (preincubation: 30 μM, 30 min) and untreated slices of the rabbit hippocampus; (B) in the presence of yohimbine (throughout superfusion)

| *8-Phth at $S_2$ (10 μM)* | *NEM-treated* | *Yohimbine* | *$S_2/S_1$* | *% increase* | n |
|---|---|---|---|---|---|
| A | | | | | |
| − | − | − | 1.00 ± 0.02 | − | 13 |
| + | − | − | 1.15 ± 0.03† | 15 | 4 |
| − | + | − | 1.00 ± 0.02 | − | 8 |
| + | + | − | 1.02 ± 0.03 | 2 | 4 |
| B | | | | | |
| − | − | + | 1.01 ± 0.02 | − | 4 |
| + | − | + | 1.19 ± 0.02** | 18 | 4 |

During superfusion the slices were stimulated twice electrically at 60 ($S_1$) and 125 min ($S_2$). Cocaine (30 μM) was present throughout superfusion in all experiments; 8-phenyltheophylline was added 15 min before $S_2$. $S_2/S_1$ and % increase compared to the respective control group are given. $S_1$ (expressed as % of tissue tritium ± s.e.mean) of drug-free slices was 3.86 ± 0.11% ($n = 17$), of NEM-treated slices 6.21 ± 0.20 ($n = 12$) and in the presence of yohimbine 21.64 ± 0.71 ($n = 8$).
Means ± s.e.mean of *n* observations are given; significant differences from the respective control group: $^{**}P<0.01$, $\dagger P \pm 0.0001$.

**Table 3** Antagonism of 8-phenyltheophylline (8-Phth) to the action of (−)-$N^6$-(R-phenylisopropyl)-adenosine ((−)-PIA) on the electrically-evoked overflow of tritium in N-ethylmaleimide (NEM)-treated and untreated slices of the rabbit hippocampus

| *8-Phth at $S_2$ (10 μM)* | *NEM-treated* | *$S_1$ (%)* | *$S_2$ (%)* | *$S_2-S_1$ (%)* | n |
|---|---|---|---|---|---|
| + | − | 2.44 ± 0.19 | 3.65 ± 0.25 | 1.21 ± 0.08 | 4 |
| + | + | 5.27 ± 0.14 | 6.10 ± 0.16 | 0.83 ± 0.08* | 4 |

Slices were either preincubated in NEM-containing (30 μM) or NEM-free medium for 30 min. During superfusion the slices were stimulated twice electrically at 60 ($S_1$) and 125 min ($S_2$). (−)-PIA (0.1 μM) was present throughout superfusion, in addition to cocaine (30 μM); 8-phenyltheophylline was added 15 min before $S_2$, $S_1$, $S_2$, and the differences $S_2-S_1$ (expressed as % of tissue tritium, respectively) of each group are given.
Means ± s.e.mean of *n* observations are given; significant differences from NEM-untreated slices: $^{*}P \pm 0.05$.

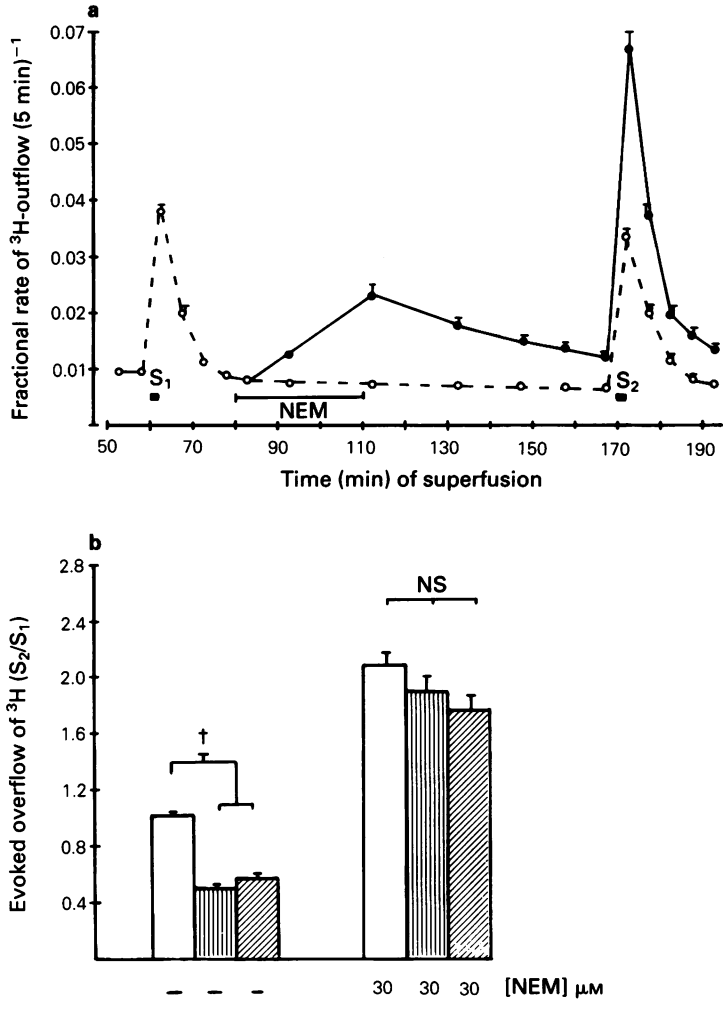


**Figure 2** (a) Effect of N-ethylmaleimide (NEM) treatment between $S_1$ and $S_2$ on basal and on electrically-evoked outflow of tritium from slices of the rabbit hippocampus incubated with [$^3$H]-noradrenaline. NEM (30 μM) was present from 80–110 min. Slices were stimulated at 60 ($S_1$) and 170 min ($S_2$) by an electrical field for 2 min each (2 ms, 3 Hz, 24 mA, 5 V cm$^{-1}$). The evoked outflow of tritium (expressed as % of tissue tritium at the onset of the respective stimulation period) was 3.81 ± 0.14% ($S_1$) or 3.74 ± 0.27% ($S_2$) for the control slices ($n = 3$; O---O and was 3.56 ± 0.24% ($S_1$) or 8.11 ± 0.37% ($S_2$, $P < 0.001$ vs. controls) for the NEM-treated slices ($n = 5$; ●——●). Fractional rates of tritium outflow are depicted as means with s.e.mean shown by vertical bars. (b) Receptor protection experiments. Experimental protocol as described above. (−)-N$^6$-(R-phenylisopropyl)-adenosine ((−)-PIA, 10 μM) was either added from 65 min onwards (15 min before NEM treatment; columns with vertical hatching) throughout the experiment or from 155 min (45 min after NEM; columns with diagonal hatching) onwards. NEM was present at a concentration of 30 μM from 80–110 min. Effects of (−)-PIA or NEM are expressed as the ratio ($S_2/S_1$) of tritium outflow evoked by the two stimulation periods. Means of the $S_2/S_1$ ratios ± s.e.mean are given, number of experiments $n = 4$–8; significance of the (−)-PIA effects: †$P < 0.0001$.

*Receptor protection experiments*

Slices of the rabbit hippocampus were treated as shown in Figure 2a. NEM (30 μM) was added between $S_1$ and $S_2$ for 30 min. NEM enhanced the basal outflow of tritium in the period from 85–165 min by 14.42 ± 2.24% of tissue tritium. The electrically-evoked overflow of tritium was increased by NEM by 105% as evident from the respective $S_2/S_1$ values which were raised from 1.02 ± 0.03 to 2.09 ± 0.12.

To study the question of whether the reduced inhibitory action of (−)-PIA may be due to a direct interaction with the agonist binding site of the receptor, receptor protection experiments were performed. In addition to NEM, (−)-PIA was present at the supramaximal concentration of 10 μM either prior to and during or following NEM treatment. The stimulus-evoked overflow of tritium was decreased by (−)-PIA to almost the same degree regardless of the duration of (−)-PIA exposure (from 65–195 or 155–195 min, respectively; Figure 2b). NEM (30 μM), present from 80–110 min, increased the stimulus-evoked overflow significantly and abolished the effect of (−)-PIA (Figure 2b). The attenuation of the (−)-PIA effect by NEM was not significantly altered when the $A_1$-receptors were occupied by (−)-PIA prior to and during the NEM treatment.

*Interaction between the $A_1$-receptor and the $\alpha_2$-adrenoceptor*

The inhibitory action of (−)-PIA on the stimulation-evoked overflow of tritium from slices of the rabbit hippocampus was investigated in the presence of the $\alpha_2$-adrenoceptor antagonist, yohimbine. Yohimbine 0.1 μM, alone, increased the evoked tritium overflow by about 200%, yohimbine 1 μM by about 400%. The inhibitory action of (−)-PIA was significantly enhanced in the presence of yohimbine (Figure 3). (−)-PIA 1 μM, given alone, reduced the electrically-evoked overflow of tritium by 44%, in the presence of yohimbine 0.1 μM, by 56% and at yohimbine 1 μM, by 72%.

The inhibitory action of endogenous adenosine was not significantly enhanced in the presence of yohimbine 1 μM. 8-Phenyltheophylline, alone, as well as in the presence of yohimbine, increased the evoked tritium overflow by about 15% (Table 2).

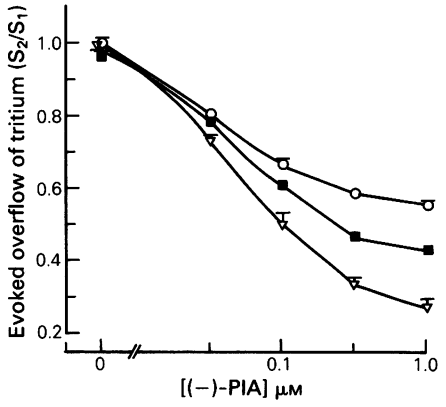


**Figure 3** Effect of (−)-$N^6$-(R-phenylisopropyl)-adenosine ((−)-PIA) on the evoked overflow of tritium in the presence of yohimbine. Slices were labelled with [$^3$H]-noradrenaline, superfused and stimulated twice electrically. (−)-PIA was added to the superfusion medium from 15 min before $S_2$ up to the end of the experiment. Yohimbine (0.1, 1 μM) was present throughout superfusion. $S_1$ was 3.84 ± 0.07% of tissue tritium for control slices (○; $n = 44$), 12.92 ± 0.57% of tissue tritium ($n = 23$) in the presence of yohimbine 0.1 μM (■) and 21.39 ± 0.40% of tissue tritium ($n = 19$) for yohimbine 1 μM (▽). The effect of (−)-PIA is expressed as the ratio $S_2/S_1$ of the evoked overflow of tritium evoked by the two stimulation periods. Means of the ratios are given with s.e.mean shown by vertical bars; numbers of experiments 3–8.

## Discussion

The release of various neurotransmitters, e.g. of acetylcholine (Harms *et al.*, 1979; Pedata *et al.*, 1983; Spignoli *et al.*, 1984; Jackisch *et al.*, 1984b), noradrenaline (Harms *et al.*, 1978; Fredholm *et al.*, 1983; Jackisch *et al.*, 1985a) and 5-hydroxytryptamine (Harms *et al.*, 1979; Feuerstein *et al.*, 1985) from CNS tissue slices is inhibited by adenosine. The adenosine receptors which inhibit the stimulation-evoked release of these neurotransmitters in the rabbit hippocampus have been characterized as $A_1$-receptors (Jackisch *et al.*, 1984b; 1985a; Feuerstein *et al.*, 1985). In this tissue the existence of an inhibitory tone of endogenous adenosine on neurotransmitter release has been postulated from experiments with the adenosine receptor antagonist, 8-phenyltheophylline and with adenosine deaminase, which metabolizes adenosine to inactive inosine (Fredholm *et al.*, 1983; Jackisch *et al.*, 1984b; 1985a; Feuerstein *et al.*, 1985). However, in noradrenergic nerve terminals of the rabbit hippocampus, inhibition of release caused by endogenous adenosine was only about 10% (Jackisch *et al.*, 1985a), whereas inhibition due to activation of the $\alpha_2$-autoreceptors by endogenous noradrenaline is much more pronounced (Jackisch *et al.*, 1984b).

The electrically-evoked overflow from slices of the rabbit hippocampus, prelabelled with [$^3$H]-noradrenaline, reflects action-potential-induced neurotrans-

mitter release (Jackisch *et al.*, 1984a). The enhancement of noradrenaline release by NEM (Figure 1a) is probably due to a partial inactivation of the autoinhibitory feedback system at the level of a regulatory N-protein (Allgaier *et al.*, 1986). Similar results have been obtained in experiments with islet-activating protein, pertussis toxin (Allgaier *et al.*, 1985), which activates $N_i$ of adenylate cyclase as well as $N_o$ by ADP-ribosylation (Hildebrandt *et al.*, 1983; Sternweis & Robishaw, 1984).

All experiments described in this paper have been performed in the presence of cocaine which inhibits the noradrenergic uptake system. Therefore the possibility can be excluded that the release enhancement caused by NEM is due to an interaction of NEM with this monoamine uptake system.

Besides the electrically-induced neurotransmitter release, the basal outflow of tritium was enhanced by NEM (Figures 1a, 2a). Other authors noticed the same phenomenon in experiments with substances which enhance intracellular cyclic AMP levels, like membrane-permeating analogues of cyclic AMP (Wemer *et al.*, 1982) or adenylate cyclase activators (Markstein *et al.*, 1984).

Our experimental data suggest the involvement of a regulatory N-protein in the $A_1$-receptor-coupled signal transduction mechanism of noradrenergic nerve terminals of the rabbit hippocampus: (1) inhibition of noradrenaline release caused by adenosine receptor agonists was markedly reduced by NEM (Figure 1b; Table 1); this effect was even more pronounced in the presence of the $\alpha_2$-adrenoceptor antagonist, yohimbine (Figure 1c; Table 1); (2) the small but significant enhancement of noradrenaline release by the adenosine receptor antagonist 8-phenyltheophylline was not significant in NEM-treated slices (Table 2); (3) in the presence of (−)-PIA (0.1 $\mu$M), i.e. under conditions of an increased inhibitory tone, release facilitation by 8-phenyltheophylline was significantly decreased by NEM compared to the respective controls (Table 3).

The reduction of the inhibitory effect of (−)-PIA on noradrenaline release in NEM-pretreated slices, indicated by a flattening of the concentration-response curve of (−)-PIA and a diminished enhancement of noradrenaline release by 8-phenyltheophylline in the presence of (−)-PIA, is compatible with the assumption of a functional invalidation of the $A_1$-receptor-coupled signal transduction system caused by an alkylation of the N-protein by NEM. Based on the results obtained by receptor protection experiments, a direct effect of NEM on the agonist binding site can be excluded.

Recently, evidence has been presented for an involvement of a regulatory N-protein, possibly the $N_i$ of adenylate cyclase, in the mechanism which transduces extracellular signals via the adenosine receptor to intracellular responses. Dolphin & Prestwich (1985) demonstrated on cultured cerebellar neurones that IAP prevented the adenosine-receptor-mediated inhibition of forskolin-stimulated adenylate cyclase. In the same system the (−)-PIA-induced inhibition of [$^3$H]-glutamate release was altered to facilitation. NEM pretreatment of rat fat cells inhibited the binding of (−)-[$^3$H]-PIA, whereas binding of adenosine receptor antagonists was not affected. In addition, the (−)-PIA-induced inhibition of adenylate cyclase was attenuated (Ukena *et al.*, 1984). Analogous effects of NEM on agonist and antagonist binding and inhibition of adenylate cyclase were obtained in rat hippocampal slices (Yeung & Green, 1983; Fredholm *et al.*, 1985). Fredholm & Lindgren (1986) reported that the PIA-inhibited release of noradrenaline from rat hippocampal slices was abolished by NEM. They concluded, however, from results with the adenylate cyclase activator, forskolin and SQ 22536, an inhibitor of adenylate cyclase, that the receptor-coupled N-protein is possibly linked to an effector other than adenylate cyclase.

From these data it cannot be decided which regulatory N-protein, the $N_i$ of adenylate cyclase or perhaps $N_o$, is involved in the $A_1$-receptor coupled signal transduction system. No determinations of NEM-dependent changes in the cyclic AMP content of hippocampal slices have been performed. Presynaptically formed cyclic AMP most probably represents only a small fraction of the total cyclic AMP content of the slice. Therefore, it seems to be extremely doubtful to correlate alterations of the cyclic AMP content of the slice with presynaptic events.

In the presence of the $\alpha_2$-adrenoceptor antagonist yohimbine, which increased noradrenaline release by autoreceptor blockade, the inhibitory action of (−)-PIA was markedly enhanced. However, this effect became apparent only if the inhibitory action of (−)-PIA was more than 20% (Figure 3). For this reason it is comprehensible that the inhibitory effect of endogenous adenosine was not (visibly) enhanced by autoreceptor blockade (Table 2). The increased action of (−)-PIA in the presence of yohimbine indicates that a common mechanism is involved in the modulation of noradrenaline release via the $A_1$- and the autoreceptor. The level at which this interaction takes place is unknown; it may be at the receptor level or at a later step in the signal transduction mechanisms. Jackisch *et al.* (1986) reported that the inhibition of noradrenaline release by κ-opioid receptors is also enhanced in the presence of yohimbine. Ramme (1986) demonstrated on the sympathetic axons of rabbit jejunal arteries that the mere blockade of the $\alpha_2$-adrenoceptors by yohimbine is sufficient to make the otherwise quiescent κ-receptors operative. However, in view of the results obtained with NEM in the case of the $A_1$- as well as of the $\alpha_2$-autoreceptor (Allgaier *et al.*, 1986) it seems reasonable to assume the existence of a common

regulatory N-protein, whose availability for coupling with the activated $A_1$-receptor may be dependent on the extent of autoreceptor occupation. The finding that in NEM-treated slices the potency of (−)-PIA to inhibit noradrenaline release was independent of the presence of yohimbine may be explained by the assumption that NEM inactivates a certain fraction of the regulatory N-protein and supports the idea of a common N-protein. However, the possibility cannot be excluded that both mechanisms, the $A_1$-receptor-coupled as well as the autoreceptor-coupled one, will meet each other at a later step of their signal transduction mechanism, possibly at the level of a common $Ca^{2+}$-channel. Recently, it has been reported that neuronal $\alpha_2$-adrenoceptors may be coupled via a regulatory N-protein to those ionic channels (Holz *et al.*, 1986).

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## References


ALLGAIER, C., FEUERSTEIN, T.J., JACKISCH, R. & HERTTING, G. (1985). Islet-activating protein (pertussis toxin) diminishes $\alpha_2$-adrenoceptor mediated effects on noradrenaline release. *Naunyn-Schmiedebergs Arch. Pharmac.*, **331**, 235–239.

ALLGAIER, C., FEUERSTEIN, T.J. & HERTTING, G. (1986). N-ethylmaleimide (NEM) diminishes $\alpha_2$-adrenoceptor mediated effects on noradrenaline release. *Naunyn-Schmiedebergs Arch. Pharmac.*, **333**, 104–109.

ASANO, T. & OGASAWARA, N. (1986). Uncoupling of γ-aminobutyric acid B receptors from GTP-binding proteins by N-ethylmaleimide: Effect of N-ethylmaleimide on purified GTP-binding proteins. *Molecular Pharmac.*, **29**, 244–249.

BOYER, J.L., GARCIA, A., POSADAS, C. & GARCIA-SAINZ, J.A. (1984). Differential effect of pertussis toxin on the affinity state for agonists of renal $\alpha_1$- and $\alpha_2$-adrenoceptors. *J. biol. Chem.*, **259**, 8076–8079.

BRUNS, R.F., DALY, J.W. & SNYDER, S.H. (1983). Adenosine receptor binding: Structure-activity analysis generates extremely potent xanthine antagonists. *Proc. natn. Acad. Sci. U.S.A.*, **80**, 2077–2080.

COCKCROFT, S. & GOMPERTS, B.D. (1985). Role of guanine nucleotide binding protein in the activation of polyphosphoinositide phosphodiesterase. *Nature*, **314**, 534–536.

COOPER, D.M.F. (1982). Bimodal regulation of adenylate cyclase. *FEBS Lett.*, **138**, 157–163.

COTE, T.E., FREY, E.A. & SEKURA, R.D. (1984). Altered activity of the inhibitory guanyl nucleotide-binding component ($N_i$) induced by pertussis toxin. Uncoupling of $N_i$ from receptor with continued coupling of $N_i$ to the catalytic unit. *J. biol. Chem.*, **259**, 8693–8698.

DOLPHIN, A.C. & PRESTWICH, S.A. (1985). Pertussis toxin reverses adenosine inhibition of neuronal glutamate release. *Nature*, **316**, 148–150.

FEUERSTEIN, T.J., HERTTING, G. & JACKISCH, R. (1985). Modulation of hippocampal serotonin (5-HT) release by endogenous adenosine. *Eur. J. Pharmac.*, **107**, 233–242.

FREDHOLM, B.B., JONZON, B. & LINDSTRÖM, K. (1983). Adenosine receptor mediated increases and decreases in cyclic AMP in hippocampal slices treated with forskolin. *Acta physiol. scand.*, **117**, 461–463.

FREDHOLM, B.B., LINDGREN, E. & LINDSTRÖM, K. (1985). Treatment with N-ethylmaleimide selectively reduces adenosine receptor-mediated decreases in cyclic AMP accumulation in rat hippocampal slices. *Br. J. Pharmac.*, **86**, 509–513.

FREDHOLM, B.B. & LINDGREN, E. (1986). Possible involvement of the $N_i$-protein in the prejunctional inhibitory effect of a stable adenosine analogue (R-PIA) on noradrenaline release in the rat hippocampus. *Acta physiol. scand.*, **126**, 307–309.

GILMAN, A.G. (1984). G proteins and dual control of adenylate cyclase. *Cell*, **36**, 577–579.

HARDEN, T.K., SCHEER, A.G. & SMITH, M.M. (1982). Differential modification of the interaction of cardiac muscarinic cholinergic and beta-adrenergic receptors with a guanine nucleotide binding component(s). *Molec. Pharmac.*, **21**, 570–580.

HARMS, H.H., WARDEH, G. & MULDER, A.H. (1978). Adenosine modulates depolarization-induced release of $^3$H-noradrenaline from slices of rat brain neocortex. *Eur. J. Pharmac.*, **49**, 305–308.

HARMS, H.H., WARDEH, G. & MULDER, A.H. (1979). Effects of adenosine on depolarization-induced release of various radiolabelled neurotransmitters from slices of rat corpus striatum. *Neuropharmac.*, **18**, 577–580.

HILDEBRANDT, J.D., SEKURA, R.D., CODINA, J., IYENGAR, R., MANCLARK, C.R. & BIRNBAUMER, L. (1983). Stimulation and inhibition of adenylyl cyclases mediated by distinct regulatory proteins. *Nature*, **302**, 706–709.

HOLZ, G.G., RANE, S.G. & DUNLAP, K. (1986). GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature*, **319**, 670–672.

JACKISCH, R., WERLE, E. & HERTTING, G. (1984a). Identification of mechanisms involved in the modulation of release of noradrenaline in the hippocampus of the rabbit in vitro. *Neuropharmac.*, **23**, 1363–1371.

JACKISCH, R., STRITTMATTER, H., KASAKOV, L. & HERTTING, G. (1984b). Endogenous adenosine as modulator of hippocampal acetylcholine release. *Naunyn-Schmiedebergs Arch. Pharmac.*, **327**, 319–325.

JACKISCH, R., FEHR, R. & HERTTING, G. (1985a). Adenosine: An endogenous modulator of hippocampal noradrenaline release. *Neuropharmac.*, **24**, 499–507.

JACKISCH, R., MOLL, S., FEUERSTEIN, T.J. & HERTTING, G. (1985b). Dopaminergic modulation of hippocampal noradrenaline release. Evidence for $\alpha_2$-antagonistic effects of some dopamine receptor agonists and antagonists. *Naunyn-Schmiedebergs Arch. Pharmac.*, **330**, 105–113.

JACKISCH, R., GEPPERT, M. & ILLES, P. (1986). Characterization of opioid receptors modulating noradrenaline release in the hippocampus of the rabbit. *J. Neurochem.*, **46**, 1802–1810.

JAKOBS, K.H., LASCH, P., MINUTH, M., AKTORIES, K. &

SCHULTZ, G. (1982). Uncoupling of α-adrenoceptor-mediated inhibition of human platelet adenylate cyclase by N-ethylmaleimide. *J. biol. Chem.*, **257**, 2829–2833.

KUROSE, H. & UI, M. (1983). Functional uncoupling of muscarinic receptors from adenylate cyclase in rat cardiac membranes by the active component of islet-activating protein, pertussis toxin. *J. cyclic Nucleotide Res.*, **9**, 305–318.

MARKSTEIN, R., DIGGES, K., MARSHALL, N.R. & STARKE, K. (1984). Forskolin and the release of noradrenaline in cerebrocortical slices. *Naunyn-Schmiedebergs Arch. Pharmac.*, **325**, 17–24.

MURAYAMA, T. & UI, M. (1983). Loss of the inhibitory function of the guanine nucleotide regulatory component of adenylate cyclase due to its ADP ribosylation by islet-activating protein, pertussis toxin, in adipocyte membranes. *J. biol. Chem.*, **258**, 3319–3326.

NEER, E.J., LOK, J.M. & WOLF, L.G. (1984). Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylate cyclase. *J. biol. Chem.*, **259**, 14222–14229.

NOMURA, Y., KITAMURA, Y. & SEGAWA, T. (1985). Decrease of clonidine binding affinity to $\alpha_2$-adrenoceptor by ADP-ribosylation of 41,000-dalton proteins in rat cortical membranes by islet-activating protein. *J. Neurochem.*, **44**, 364–369.

PEDATA, F., ANTONELLI, T., LAMBERTINI, L., BEANI, L. & PEPEU, G. (1983). Effect of adenosine, adenosine triphosphate, adenosine deaminase, dipyridamole and aminophylline on acetylcholine release from electrically-stimulated brain slices. *Neuropharmac.*, **22**, 609–614.

QUENNEDEY, M.C., BOCKAERT, J. & ROUOT, B. (1984). Direct and indirect effects of sulfhydryl blocking agents on agonist and antagonist binding to central $\alpha_1$- and $\alpha_2$-adrenoceptors. *Biochem. Pharmac.*, **33**, 3923–3928.

RAMME, D. (1986). Does the blockade of $\alpha_2$-adrenoceptors unmask previously silent opioid κ-receptors? *Naunyn-Schmiedebergs Arch. Pharmac.*, **332**, R80.

RODBELL, M. (1980). The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature*, **284**, 17–22.

SPIGNOLI, G., PEDATA, F. & PEPEU, G. (1984). $A_1$ and $A_2$ adenosine receptors modulate acetylcholine release from brain slices. *Eur. J. Pharmac.*, **97**, 341–342.

STERNWEIS, P.C. & ROBISHAW, J.D. (1984). Isolation of two proteins with high affinity for guanine nucleotides from membranes of bovine brain. *J. biol. Chem.*, **259**, 13806–13813.

UKENA, D., POESCHLA, E., HÜTTERMANN, E. & SCHWABE,U. (1984). Effects of N-ethylmaleimide on adenosine receptors of rat fat cells and human platelets. *Naunyn-Schmiedebergs Arch. Pharmac.*, **327**, 247–253.

WEMER, J., SCHOFFELMEER, A.N.M. & MULDER, A.H. (1982). Effects of cyclic AMP analogues and phosphodiesterase inhibitors on $K^+$-induced $^3$H-noradrenaline release from rat brain slices and on its presynaptic α-adrenergic modulation. *J. Neurochem.*, **39**, 349–356.

YEUNG, S.M.H. & GREEN, R.D. (1983). Agonist and antagonist affinities for inhibitory adenosine receptors are reciprocally affected by 5′-guanylylimidodiphosphate or N-ethylmaleimide. *J. biol. Chem.*, **258**, 2334–2339.

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