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Treatment with N-ethylmaleimide selectively reduces adenosine receptor-mediated decreases in cyclic AMP accumulation in rat hippocampal slices

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1 N-ethylmaleimide (NEM) has been reported to interact with the GTP-binding N_i-protein; we have examined its effect on adenosine receptor binding in feline cortical membranes and on adenosine-receptor mediated effects on cyclic AMP accumulation in rat hippocampal slices.

2 Treatment of cortical membranes with NEM (100 μM for 5 min) altered the binding of [^{3}H]-phenylisopropyladenosine (PIA) from being almost exclusively to a single class of high affinity sites (K_D = 1.65 nM) to binding at two classes of sites (K_{DH} = 2.1 nM, K_{DL} = 102 nM). The total number of binding sites was similar (825–845 fmol mg^{-1} in control membranes, 944–1428 fmol mg^{-1} in NEM-treated membranes).

3 In rat hippocampal slices treated with forskolin (0.3 μM) L-PIA produced a biphasic effect on cyclic AMP accumulation: an inhibition at 0.03 to 1 μM and at higher concentrations, a stimulation. Treatment with 50 μM NEM selectively inhibited the inhibitory phase, causing stimulation at lower concentrations of L-PIA. At 50 μM, NEM did not alter basal or forskolin-stimulated cyclic AMP accumulation but at higher concentrations inhibition was observed.

4 It is concluded that NEM can, in certain doses, selectively block adenosine A_1-receptor-mediated effects without affecting A_2-receptor-mediated actions in the same tissue. It is suggested that this is due to NEM affecting the N_i guanine nucleotide binding protein.

Introduction

Adenosine is able either to stimulate or inhibit adenylate cyclase. These effects are mediated via two different classes of receptors R_a or R_i, respectively (Londos *et al.*, 1980). More recently another terminology has been more widely accepted to denote the two subtypes since it does not imply a specific mechanism of action (for discussion see Stone, 1985): the two receptor types are called A_1 (essentially equivalent to R_i) and A_2 (essentially equivalent to R_a). Both forms of the receptor have been demonstrated in the rat hippocampus by binding techniques and adenylate cyclase measurements (Yeung & Green, 1983; 1984) as well as by studies of cyclic AMP accumulation in forskolin-treated slices (Fredholm *et al.*, 1983). Forskolin is a diterpene that can stimulate adenylate cyclase independently of receptor activation (Seamon & Daly, 1981).

The sulphydryl alkylating agent N-ethylmaleimide (NEM) has been shown to inhibit certain hormone-induced inhibitory responses, including α-adrenergic, muscarinic, cholinergic, histamine and TRH-mediated while leaving stimulatory effects unaltered or much less affected (Jakobs *et al.*, 1982; Harden *et al.*, 1982; Carman-Kran, 1984; Sharif & Burt, 1984; Yamaguchi *et al.*, 1984). In membranes from NG108-15 neuroblasoma × glioma cells treatment with NEM (100–220 μM) led to a loss of the capacity of three different types of agonists (muscarinic, cholinoceptor, opiate receptor and α_2-adrenoceptor) to inhibit adenylate cyclase (Smith & Harden, 1984). There was a parallel loss of GTP-sensitive agonist binding. By contrast the capacity of prostaglandins to stimulate adenylate cyclase was unimpaired.

Recently, Yeung & Green (1983) reported that NEM-treatment caused a marked reduction in the affinity of the adenosine A_1-receptor agonist, cyclohexyladenosine (CHA) to specific binding sites in the rat hippocampus without any concomitant loss in the binding of an antagonist. The effect of NEM on adenosine receptors was further elucidated by Ukena and coworkers (1984) using membranes from rat fat cells (A_1) or blood platelets (A_2). They found that

treatment of fat cell membranes with NEM converted the high affinity binding sites for L-phenylisopropyladenosine (L-PIA) to a low affinity form, without any concomitant change in the binding of an antagonist. In low concentrations NEM reduced the ability of L-PIA to inhibit adenylate cyclase without affecting basal or isoprenaline-stimulated enzyme activity. By contrast the binding of N-ethylcarboxamido adenosine (NECA) to presumably A_2 adenosine receptors in platelets was enhanced rather than reduced by NEM treatment (Ukena *et al.*, 1984). All these results are compatible with the theory that NEM, in a limited concentration range, causes a relatively selective inactivation of the N_i GTP-binding protein that mediates the inhibitory effect of receptor agonists on adenylate cyclase and that modifies their binding.

Given this background, we wanted to see if NEM could be used in a tissue slice, containing several cell types, to antagonize the inhibitory effects of L-PIA on cyclic AMP accumulation without altering its stimulatory effects. We have also examined the effect of NEM treatment on the specific binding of L-PIA to feline cortical membranes.

Methods

For these experiments we used male Sprague-Dawley rats (150–250 g) or mongrel cats (2.5–3.5 kg).

The cats were killed by an overdose of sodium pentobarbitone or by air embolism after light anaesthesia with sodium pentobarbitone. The brains were removed and the cortical tissue dissected out. The cortices were frozen in liquid nitrogen and stored at − 80°C for a period of not more than 2 weeks.

The rats were killed by decapitation and the brains rapidly removed on ice. The hippocampus was dissected out and washed in ice-cold saline.

Formation of [^{3}H]-cyclic AMP

The middle half to one third of the hippocampus was cut transversely into 0.4 mm thick slices with a McIlwain tissue chopper. The slices were preincubated and labelled as described previously (Fredholm *et al.* 1982). All incubations were carried out in Krebs Ringer bicarbonate buffer of the following composition (mM): NaCl 118, KCl 4.85, $MgSO_4$ 1.15, KH_2PO_4 1.15, $CaCl_2$ 2.5, $NaHCO_3$ 25, glucose 11.1 and rolipram 0.03. The incubations were carried out at 37°C for 15 min with additions as indicated. Under most circumstances forskolin (0.3 μM) was added to raise the level of cyclic AMP accumulation. At the end of the experiment the slices were homogenized in 8% trichloroacetic acid and [^{3}H]-cyclic AMP was isolated by combined alumina and Dowex 50 chromatography as described by Salomon (1979) from the protein-free supernatants after centrifugation. The results are expressed as percentage conversion of radioactivity to cyclic AMP. No corrections were made for incomplete recovery (40–70%).

Preparation of brain membranes

The frozen brain tissue was crushed and homogenized in 0.25 M sucrose containing 5 mM Tris-Cl, pH 7.4, 2 mM $MgCl_2$ and 1 mM EDTA. The crude homogenate was centrifuged at 2000 *g* for 15 min. The supernatant was centrifuged at 10 000 *g* for 15–30 min. The pellet was resuspended in the same buffer and washed twice. The final pellet was resuspended in 100 mM Tris-acetate pH 7.4 containing 2 mM MgCl and 1 mM EDTA. The resuspended particulate fraction was incubated with adenosine deaminase (Boehringer, Mannheim) in a concentration of 1 μg ml^{-1} for 15 min at 30°C and thereafter stored frozen in aliquots until required. This enzyme treatment is necessary to remove endogenous ligands from the membrane preparations and does not otherwise distort the results as discussed previously (Bruns *et al.*, 1980; Schwabe & Trost, 1980). Three different preparations of pooled cat cortical membranes were used in these studies. Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin used as standard.

Binding assays

Membranes from 5 to 50 mg tissue (0.03–0.4 mg protein) were incubated in a total volume of 0.3 to 1 ml 50 mM Tris-Cl pH 7.4 and other additions as indicated. Usually incubations were carried out at 30°C for 90 to 120 min and in triplicate. The incubations were ended by filtration through Whatman GF/B filters followed by washing twice with 5 ml cold medium. The filters were removed and allowed to equilibrate for at least 12 h with the scintillation fluid before counting in a scintillation counter with an efficiency of 30–50%. Non-specific binding was determined in the presence of 1 to 10 μM L-PIA or 3 μM 2-CA and generally amounted to about 5%.

The data were analysed by microcomputer procedures. Equilibrium binding data were analysed by the LIGAND programme of Munson & Rodbard (1980) as modified for the IBM PC by McPherson (1983).

Chemicals

[2,8-^{3}H](−)-N^6-2-phenylisopropyladenosine (17 Ci mmol^{-1}), [2-^{3}H]-adenine (25 Ci mmol^{-1}) and [8-^{3}H]-adenosine 3′,5′-monophosphate, ammonium salt (28 Ci mmol^{-1}) were obtained from the Radiochemical Centre, Amersham U.K. Adenosine, aden-

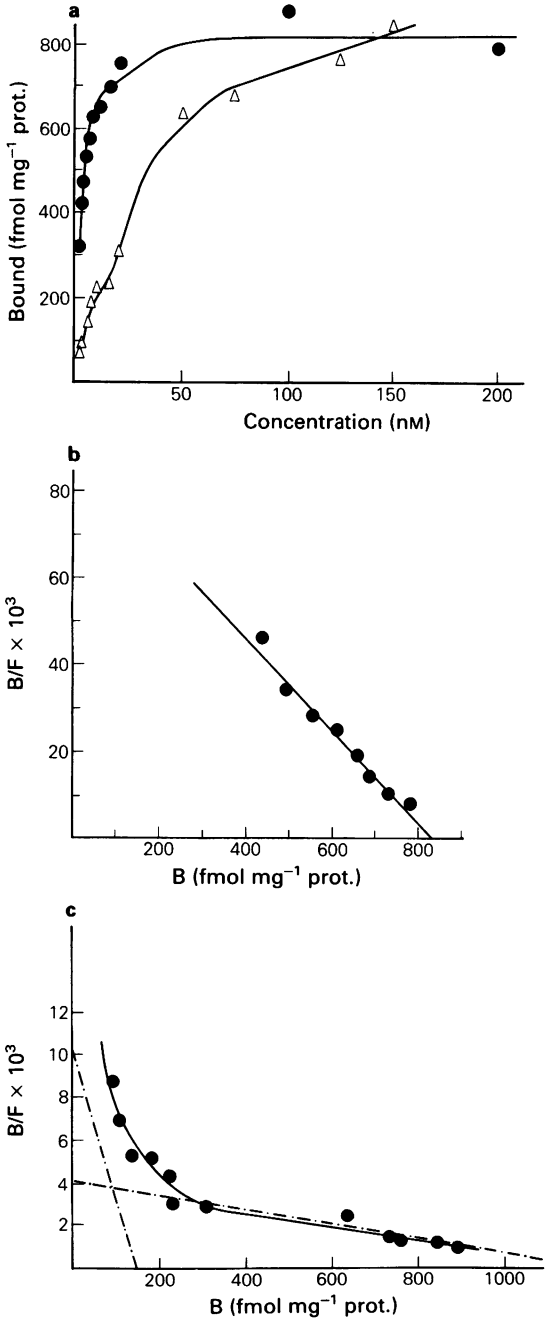

Figure 1 The effect of N-ethylmaleimide (NEM)-treatment on the binding of L-phenylisopropyladenosine (L-PIA) to feline cortical membranes. The membranes were incubated with 100 μM NEM for 5 min at 37°C. After dilution with buffer the membranes were used in the binding assay as described under Methods. (a) Shows the original binding data: (Δ) NEM-treated; (●) control; (b) and (c) give the Scatchard plots for the control and NEM-treated mumbranes, respectively. The computer-generated curves are also drawn.

osine 3',5'-monophosphate, N-ethylmaleimide were obtained from Sigma, St Louis. Mo. U.S.A. Forskolin and 8-phenyltheophylline were obtained from Calbiochem-Behring, LaJolla, Ca, U.S.A. The two diastereoisomers of PIA were obtained from Boehringer, Mannheim, BRG, Rolipram (ZK 62,711, 4-(3-cyclopentyloxy-methoxyphenyl)-2-pyrrolidone was a gift from Schering AG, Berlin, BRG.

Results

As shown in Figure 1 treatment of feline cortical membranes with NEM markedly altered the binding of L-PIA. The inhibition of binding that occurred at low agonist concentrations could be explained by a shift in the binding sites from a high affinity state to a low affinity state. After treatment with 0.1 mM NEM for 5 min only 8% of the binding sites were in the high affinity form. The total number of binding sites was not significantly altered. The fact that the total number of binding sites tended to be lower in the control incubations is probably due to the presence of a small fraction of low affinity binding sites also in this condition, which could not be analysed with certainty.

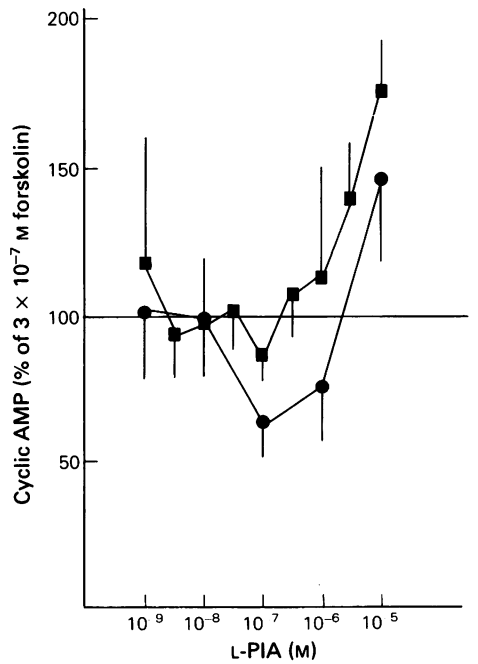

Figure 2 The effect of increasing concentrations of L-phenylisopropyladenosine (L-PIA) on the accumulation of cyclic AMP in forskolin (0.3 μM)-treated rat hippocampal slices before (●) or after treatment with N-ethylmaleimide (50 μM) (■).

Table 1 The effect of increasing doses of N-ethylmaleimide (NEM) on basal and forskolin (0.3 μM)-stimulated cyclic AMP accumulation in rat hippocampal slices

Drug treatment	*Basal*	*Forskolin*
Control (9)	0.48 ± 0.02	2.12 ± 0.15
NEM (0.05 mM) (6)	0.48 ± 0.04	2.25 ± 0.30
NEM (0.1 mM) (3)	0.44 ± 0.03	1.80 ± 0.35
NEM (0.5 mM) (3)	0.45 ± 0.10	0.41 ± 0.04
NEM (1 mM) (4)	0.36 ± 0.08	0.36 ± 0.02
NEM (3 mM) (3)	0.89 ± 0.18	0.52 ± 0.02

The slices were treated with NEM at the concentration indicated for 15 (1 and 3 mM) or 25 (0.05–0.5 mM) min. Results are expressed as percental conversion of labelled adenine nucleotides to labelled cyclic AMP. Mean ± s.d. Number of experiments are given in parentheses.

In agreement with our previous findings (Fredholm *et al.*, 1983; 1985) L-PIA had a biphasic effect on cyclic AMP accumulation in forskolin-treated rat hippocampal slices; at low concentrations it produced inhibition, at higher concentrations (above 1 μM) stimulation. Both phases were antagonized by 10 μM 8-phenyltheophylline (not shown), indicating that they are receptor-mediated. In the presence of 50 μM NEM only the stimulatory phase remained. Moreover stimulation tended to occur at lower concentrations of L-PIA than it did in the absence of NEM (Figure 2). At higher concentrations of NEM the stimulatory effect of L-PIA was also affected (Table 1). Thus, at 500 μM NEM the stimulatory effect of forskolin was completely abolished.

Discussion

The major finding of the present study is that a low concentration of NEM can antagonize relatively selectively the inhibitory effect of L-PIA on cyclic AMP accumulation in rat hippocampal slices. This inhibitory effect is probably mediated via adenosine receptors of the A_1 subtype (Fredholm *et al.*, 1983; Fredholm, Jonzon & Lindgren, unpublished). The stimulatory effect on cyclic AMP accumulation that is observed with higher concentrations of L-PIA and that is probably mediated via A_2-receptors was essentially unaffected by NEM at 50 μM. Similarly, this concentration of NEM did not alter the basal or the forskolin-stimulated accumulation of cyclic AMP.

As mentioned in the introduction there is good evidence that NEM binds to and modifies the N_i protein that couples receptors to inhibitory effects on the adenylate cyclase. We have confirmed in brain membranes the finding of Ukena *et al.* (1984) in fat cell membranes that NEM causes a shift in the receptor population from a high to a low affinity state. The results of Yeung & Green (1983) and of Ukena *et al.* (1984) showing that the binding of an antagonist is unaffected by NEM binding, strongly argues against NEM inactivating the receptor directly. The finding that the total number of agonist binding sites is unaltered (or at least not decreased) by NEM-treatment similarly argues against an inactivation at the level of the receptor (Yeung & Green, 1983; Ukena *et al.*, 1984; present results). If it is assumed that there is a small component of low affinity binding also in the control situation, which was not detected by us, and that the total number of binding sites is, in fact, identical in both instances then it can be calculated that 70% of the binding sites are in the high affinity state in the control situation as compared to 8% after NEM-treatment. These figures agree very well with those reported by Ukena *et al.* (1984).

The present results therefore suggest that NEM can be used as a tool, even in a relatively complex preparation such as the hippocampal since preparation, to discriminate between such receptor-mediated events as are mediated via the N_i protein and those that are not. In this respect NEM may be used as a complement to the islet-activating protein (IAP), isolated from pertussis toxin by Ui and coworkers (1984). NEM is unlikely to act completely selectively but has the advantage of producing its effects rapidly. By contrast IAP is probably quite specific but may require a long time to produce complete inactivation of the N_i protein. In particular NEM and IAP may be used to determine which effects of adenosine are mediated via the N_i-protein. These effects may or may not all be secondary to changes in adenylate cyclase and cyclic AMP formation.

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