MOLECULAR PHARMACOLOGY, 28:155–162

N-Ethylmaleimide-Induced Changes in Agonist Affinity for Histamine H_1-Receptors in the Guinea Pig Brain

EDOUARD YERAMIAN, MONIQUE GARBARG, AND JEAN-CHARLES SCHWARTZ

Unité 109 de Neurobiologie, Centre Paul Broca de l'Institut National de la Santé et de la Recherche Médicale, 2 ter rue d'Alésia, 75014 Paris, France

Received January 29, 1985; Accepted May 23, 1985

SUMMARY

The effect of the thiol-alkylating agent, *N*-ethylmaleimide (NEM), on histamine (HA) H_1-receptors from guinea pig cerebellum, labeled with [^{3}H]mepyramine, was investigated. The properties of [^{3}H]mepyramine binding (apparent dissociation constant and maximal number of sites) were not modified by prior treatment of the membranes with 2 or 5 mM NEM. This treatment did not change either the inhibition curves of *d*-chlorpheniramine or mianserin, two H_1-receptor antagonists. In contrast, treatments of membranes with NEM significantly decreased the IC_{50} values of HA and the slope indexes (pseudo Hill coefficients) of HA inhibition curves, which became inferior to unity. These effects were irreversible, and their extent related to the NEM treatment duration and the NEM concentration. A computer analysis of the data indicated that part of the H_1-receptors were converted from a state of low affinity for the amine (IC_{50} value of 75 μM) into a high agonist affinity state (IC_{50} value of 2 μM). The change was less marked for partial agonists than for HA. The NEM-induced change was observed in the presence and in the absence of Na^+ ions, known to decrease the affinity of HA for H_1-receptors. Agonists or antagonists did not protect against the modification of HA affinity induced by NEM. The digitonin-solubilized receptors retained their sensitivity to NEM. Among other thiol reagents, iodoacetamide and iodoacetic acid were ineffective, and organic mercurial agents strongly reduced the number of [^{3}H]mepyramine-binding sites. NEM treatment might alkylate a critical thiol group located outside the ligand-binding domain of the H_1 receptor and thereby stabilize the latter in a conformation distinct from that of the activated state.

INTRODUCTION

Very little is known about the molecular properties, effector and regulatory mechanisms, of histamine H_1-receptors as compared with many other hormone or neurotransmitter receptors. For instance, it has been proposed that the stimulation of H_1-receptors is associated with a Ca^{2+} translocation process (1). Although this may account for various responses to HA,[1] like stimulation of smooth muscle contraction, potentiation of the stimulation of other receptors linked to stimulation of adenylate cyclase (2, 3), stimulation of cyclic GMP formation (4), or phosphatidylinositol hydrolysis (5), a direct demonstration of this hypothesis is still lacking. Nevertheless, the development of a ligand-binding technique using [^{3}H]mepyramine (6–8) has paved the way for direct biochemical characterization and purification (9, 10) of H_1-receptors. The effects of a chemical modification of thiol groups on the recognition of ligands by H_1-receptors were presently investigated for two main reasons: (i) disulfide bond-reducing and thiol-alkylating reagents were found to modify the contractions of rabbit aorta to HA, an H_1-receptor-mediated response (11); (ii) thiol-modifying reagents affect the recognition of ligands, mainly agonists, by a wide variety of receptors (reviewed by Strauss (12)). For instance, the involvement of sulfhydryl groups has been proposed for muscarinic cholinergic (13), β-adrenergic (14), dopaminergic (15), and opiate (16) receptors. This study was performed on membranes from guinea pig cerebellum, a tissue with a high density of [^{3}H]mepyramine-binding sites.

[1] The abbreviations used are: HA, histamine; NEM, *N*-ethylmaleimide.

EXPERIMENTAL PROCEDURES

Preparation of cerebellar membranes. Male Hartley guinea pigs (300–400 g, generously provided by Rhône Poulenc) were used. Immediately after decapitation each cerebellum was homogenized with a Polytron in 20 ml of cold 50 mM KH_2PO_4/Na_2HPO_4 buffer, pH 7.5. The homogenate was first centrifuged at 1000 × *g* × min, and the resulting supernatant spun at $3 \cdot 10^5 \times g \times$ min in a Beckman centrifuge. The pellet was washed twice superficially with fresh buffer and kept at −80° (up to 2 months without loss of activity) before resuspension for binding experiments. For experiments in a sodium-free medium, membrane preparations were performed in 50 mM Tris-HCl buffer, pH 7.5.

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Treatment of membranes. For NEM treatment the pellets were resuspended in fresh 50 mM phosphate buffer, pH 7.5, with a Dounce homogeneizer and incubated at 25° in the presence of the reacting agent at appropriate concentration and for appropriate time. In most experiments the treatment time was 20 min. NEM solution (0.3 ml) prepared in buffer (or 0.3 ml of buffer for the control membranes) was added to 2.7-ml aliquots of the membrane suspension (about 2 mg of protein) in centrifugation tubes, which were gently shaken throughout the incubation time. The reaction was stopped by addition of 2-mercaptoethanol at a final concentration twice that of NEM. The content of each tube was then diluted by addition of 7 ml of cold buffer, and the tubes were centrifuged in a Beckman centrifuge ($3 \cdot 10^5 \times g \times$ min) once for all routine treatments. The pellets were superficially washed before they were resuspended in 5.6 ml of fresh buffer for the binding experiments.

In order to evaluate the ability of various agents (H_1-receptor agonists or antagonists) to protect against the NEM effect, four different conditions were tested: control membranes, NEM-treated membranes, control membranes preincubated with the agent, and membranes preincubated with the agent prior to NEM treatment. The resuspended pellets were incubated with the agent for 25 min at 25° under agitation. Then, NEM was added and experiments performed as previously described. In some experiments performed with a high concentration of agonists or antagonists, the pellets were resuspended and centrifuged once or twice more before binding experiments.

For iodoacetamide or iodoacetic acid treatments, the incubation time was 1 h, and the reaction was stopped as for NEM treatment. For *p*-chloromercuriphenylsulfonic acid and mersalyl treatments, the incubation time was 20 min, and the reaction was stopped only by addition of cold buffer and centrifugation, without addition of 2-mercaptoethanol. In some experiments, a washing step was included before binding experiments.

Binding experiments with membranes. Aliquots of the membrane suspension (450 μl containing about 180 μg of protein) were incubated with 50 μl of a [^{3}H]mepyramine solution (0.6 nM final concentration in routine experiments), alone or together with nonradioactive ligands in increasing concentrations. After 30 min at 25°, the reaction was stopped by dilution with 2 × 3 ml of cold buffer, followed by rapid filtration over Whatman GF/B filters under vacuum. Filters were washed with 2 × 10 ml of cold buffer, and radioactivity was measured by liquid scintillation spectrometry. Specific binding was evaluated as the difference between radioactivity bound in the absence and in the presence of 0.2 μM mianserin, an H_1-receptor antagonist. At 0.6 nM [^{3}H]mepyramine, it represented about 90% of the total binding.

Preparation, NEM treatment of, and binding experiments on solubilized receptors. Membranes (about 7 mg of protein) were resuspended in 5 ml of 50 mM KH_2PO_4/Na_2HPO_4 phosphate buffer, pH 7.5, containing 0.5% digitonin. After 1 h at 0°, the suspension was centrifuged at 80,000 × *g* (Beckman-Spinco centrifuge) for 2 h. Approximately 50% of proteins and 60% of [^{3}H]mepyramine binding sites were solubilized by this procedure.

The supernatant was diluted 2-fold with the same phosphate buffer containing no digitonin, and the NEM treatment was performed as described for membranes. After addition of mercaptoethanol, without centrifugation, 450-μl aliquots (about 160 μg of protein) were incubated for 30 min at 25° with 0.6 nM [^{3}H]mepyramine, alone or together with appropriate drugs. The specific binding represented 87%.

At the end of the incubation, 50 μl of a 10% suspension of activated charcoal in buffer containing 2% bovine serum albumin was added (9). After a 25-s agitation and a 2-min centrifugation at 10,000 × *g*, 300 μl of supernatant were taken and counted for radioactivity.

Analysis of binding data using one- or two-component models. The inhibition parameters were calculated by a computerized iterative method using either a one-component or a two-component model (17). The computerized process is based upon the conventional principle of the least squares, including the calculation of the variance-covariance matrix, the coefficients of which allowed the estimation of the standard errors on the best estimates of I_{max} and IC_{50} values.

In the one-component model the inhibition curve was fitted according to the equation,

$$B\% = \frac{B}{B_o} \times 100 = \frac{I_{max} \times I}{I + IC_{50}}$$

where B_o represents the total specific binding and B the amount of specific binding in the presence of the inhibitor at the concentration I. I_{max} is the maximally inhibited binding (ideally, in the one-component model I_{max} corresponds to 100%), and IC_{50} is the inhibitor concentration producing a $I_{max}/2$ inhibition of binding.

A slope index n_H (pseudo Hill coefficient) was calculated along with the one-component analysis; n_H represents the slope of the regression line ln $B\%/(I_{max} - B\%)$ as a function of ln I. In the two-component model, the inhibition curve was fitted according to the equation,

$$B = \frac{R_H \times I}{I + (IC_{50})_H} + \frac{R_L \times I}{I + (IC_{50})_L}$$

where the high- and low-affinity components are designated by the indices H and L, respectively, and R_H and R_L designate the respective contributions of the two components expressed as per cents of the total population.

Testing the significant resolution in two components by the two-site model was performed using a F-test as follows. F is the ratio of the deviation mean squares obtained in the one-site model and that obtained in the two-site model. The deviation mean squares represent the sum of squares of residuals divided by the number of degrees of freedom (number of data points minus number of parameters calcu-

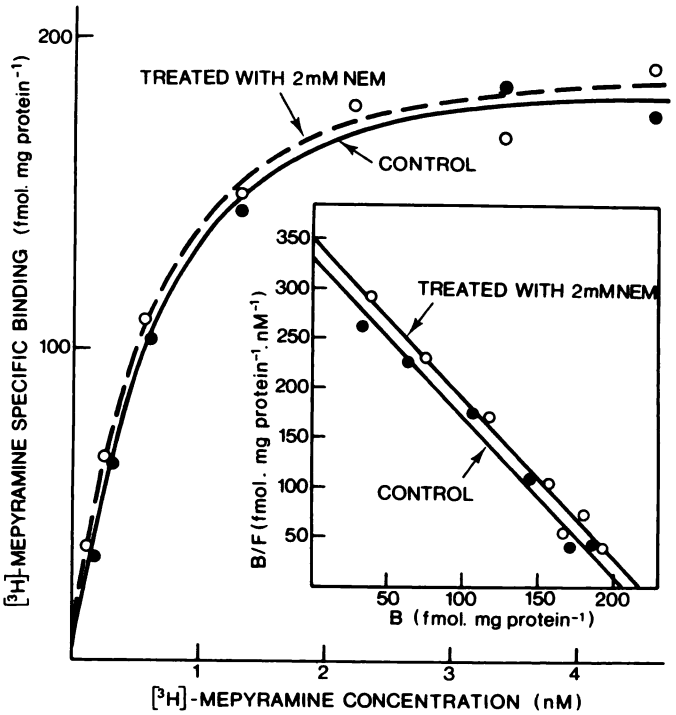

FIG. 1. *Effect of a NEM treatment on specific [^{3}H]mepyramine binding to cerebellar membranes*

Values shown are the means of six determinations in two separate experiments. Parameters of specific [^{3}H]mepyramine binding obtained by computerized analysis (27) were: B_{max} = 190 ± 13 fmol/mg protein, K_d = 0.50 ± 0.08 nM (control membranes), and B_{max} = 205 ± 16 fmol/mg of protein, K_d = 0.56 ± 0.12 nM (membranes treated with 2 mM NEM).

lated). The analysis by a two-site model was considered to be significantly improved when the F value exceeds that of F-distribution for a 1% level.

Materials. [^{3}H]Mepyramine (27 Ci/mmol) was purchased from New England Nuclear. The following were gifts: mianserin from Organon, D-chlorpheniramine and cyclizine from Burroughs-Wellcome, mepyramine from Rhône-Poulenc, betahistine from Duphar, and other H_1-receptor agonists from Smith Kline and French. All other chemicals were from Sigma.

RESULTS

Effects of NEM on [^{3}H]mepyramine binding. A saturation curve of [^{3}H]mepyramine was established on control membranes from guinea pig cerebellum and on membranes treated with 2 mM NEM. In control membranes, the analysis (17) of [^{3}H]mepyramine binding gave a K_d value of 0.50 ± 0.08 nM and a B_{max} value of 190 ± 13 fmol/mg protein (Fig. 1). For 2 mM NEM-treated membranes, the K_d value was 0.56 ± 0.12 nM and the B_{max} value 205 ± 16 fmol/mg protein. Scatchard analysis of the same data did not evidence any significant modification in the parameters of [^{3}H]mepyramine binding after 2 mM NEM treatment (*inset* of Fig. 1). Increasing the concentration of NEM to 5 mM did not result in any change of the number of specific binding sites labeled with 0.6 nM [^{3}H]mepyramine (data not shown).

Effects of NEM on the inhibition of [^{3}H]mepyramine binding by histamine. The HA inhibition curves were shifted leftward following membrane treatments with either 2 or 5 mM NEM for 20 min, the "shift factors" (ratios of mean IC_{50} values in control and treated membranes) being of 2.8 and 3.7, respectively (Figs. 2 and 5). Addition of 2-mercaptoethanol (4–10 mM) alone did not change the number of binding sites or the effect of HA. The effect of NEM was already detectable at 0.5 mM NEM and still present at 10 mM NEM (not shown). In the absence of Na^+ (Tris-HCl buffer) the HA inhibition curves of both control and 2 mM NEM-treated membranes were shifted leftward so that the shift factor was again 2.8 (Fig. 2). The NEM-induced shift was accompanied by a shallowing of the HA inhibition curves evidenced both by the curvilinear pseudo Scatchard plot (Fig. 3) and the computerized analysis (Table 1). The latter indicated that, whereas HA inhibition on control membranes occurred at a single site (IC_{50} = 78 μM), the inhibition data were best fitted ($p < 0.01$) to a two-site model on NEM-treated membranes. In this model the high affinity component (IC_{50} value about 2 μM) represented 24 and 48% of sites for 2 and 5 mM NEM, respectively, and the low affinity component displayed an IC_{50} value similar to that of the single component in control membranes (Table 1).

The effect of NEM was time dependent with $t½$ values of about 6 and 7 min for 5 and 2 mM NEM, respectively (Fig. 4). Hence, the 20-min treatment selected for most studies corresponded to a nearly maximal effect.

When the NEM treatment was performed in the presence of 25 nM *d*-chlorpheniramine, an H_1-receptor antagonist with a K_i value of 0.9 nM (18), the effect of the alkylating agent on the HA inhibition curve was not significantly modified (Fig. 5). In similar experiments, no protection against NEM was provided either using *d*-chlorpheniramine in higher concentrations (up to 150 nM), other H_1-receptor antagonists like mepyramine, mianserin, and cyclizine (up to 200 nM), or HA itself (up

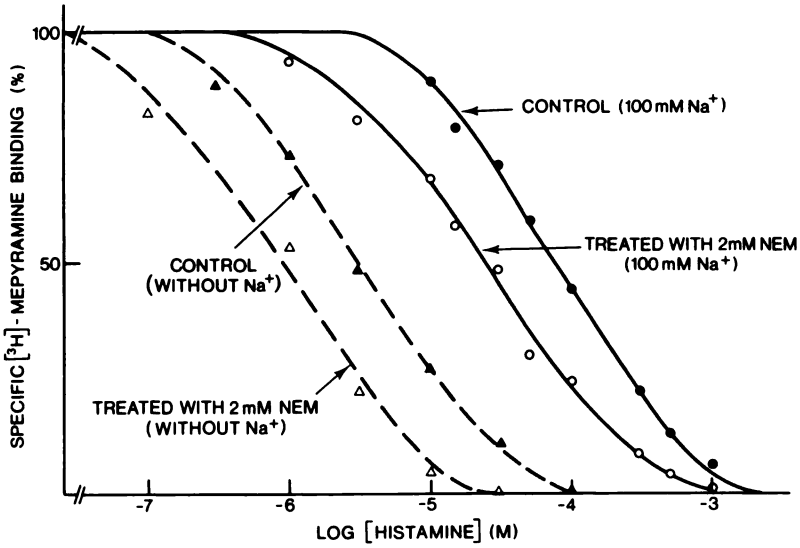

FIG. 2. *Effects of a treatment with 2 mM NEM on the inhibition by HA of [^{3}H]mepyramine binding to cerebellar membranes in the presence or in the absence of sodium ions*

Preparations of membranes and binding assays were performed either in phosphate buffer containing 100 mM Na^+ (*solid line* curves) or in Tris-HCl buffer without Na^+ (*dotted line* curves). The resuspended membranes were preincubated alone (control) or with 2 mM NEM for 20 min at 25°. The reaction was stopped by addition of 2-mercaptoethanol (at a final concentration of 4 mM) the membranes centrifuged and finally resuspended for binding assays performed with 0.6 nM [^{3}H]mepyramine (30 min at 25°). Data are expressed as per cent inhibition of specific binding. Each *point* represents the combined mean from 10 separate experiments for the *solid line* curves, and from 3 separate experiments for the *dotted line* curves (triplicate determinations). Specific binding (in fmol/mg protein) was 93 ± 6 for control membranes assayed in phosphate buffer, 92 ± 6 for NEM-treated membranes assayed in phosphate buffer, 71 ± 3 for control membranes assayed in Tris-HCl buffer, and 64 ± 3 for NEM-treated membranes assayed in Tris-HCl buffer.

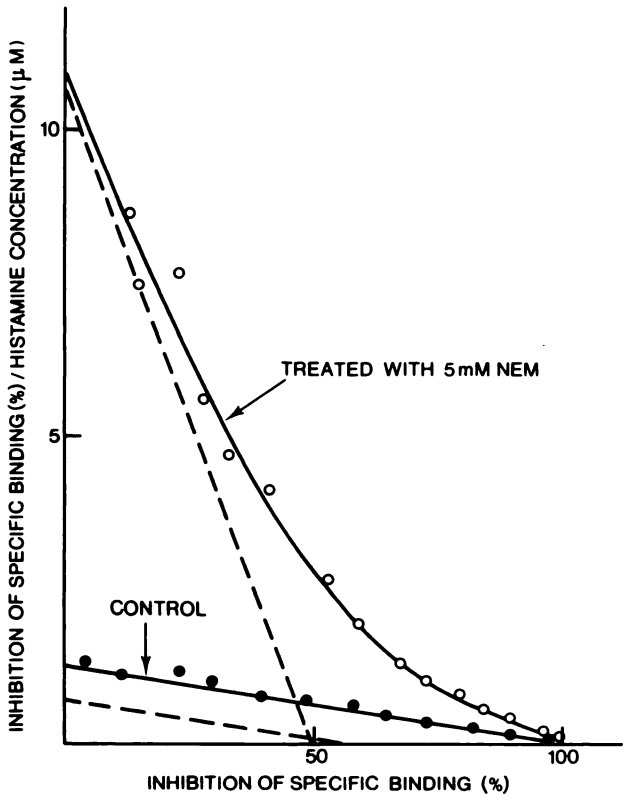

FIG. 3. *Pseudo Scatchard plots of the inhibition of [^{3}H]mepyramine binding by HA in control and in NEM-treated membranes*

Membranes were incubated with 5 mM NEM for 20 min at 25° and the reaction stopped by addition of 2-mercaptoethanol (10 mM final concentration). The data are the mean of 5 separate experiments. For the NEM-treated membranes, the *dotted lines* were drawn according to a computer analysis by an iterative method for a two-site model (see "Experimental Procedures" and Table 1). The specific binding of 0.6 nM [^{3}H]mepyramine (in fmol/mg protein) was 108 ± 2 for control membranes and 98 ± 9 for NEM-treated membranes.

TABLE 1

Analysis of the inhibition of [^{3}H]mepyramine binding by histamine in NEM-treated membranes

Inhibition parameters were calculated from data presented in Figs. 2 and 3 according to a two-site model. *R* represents the percentage of sites belonging to high and low affinity components. IC_{50} values represent the HA concentrations for half-maximal inhibition of each component.

Treatments	High affinity component		Low affinity component	
	R	IC_{50} value	*R*	IC_{50} value
	%	(μM)	%	(μM)
None			100	78 ± 3.5
NEM (2 mM)	24 ± 14	1.6 ± 1.4	76 ± 13	41 ± 13
NEM (5 mM)	48 ± 5	2.3 ± 0.4	52 ± 4	73 ± 14

to 4 mM) (not shown). In digitonin extracts of cerebellar membranes, the NEM treatment resulted in a limited decrease of [^{3}H]mepyramine binding to solubilized H_1-receptors accompanied by a leftward shift and a shallowing of the HA inhibition curve (shift factor = 4 and n_H = 0.77 instead of 1.04) (Fig. 6).

Effects of NEM on the inhibition of [^{3}H]mepyramine binding by H_1-receptor agonists and antagonists. Treatments of membranes with either 2 or 5 mM NEM (Table 2) resulted in a leftward shift of the inhibition curves of HA and, to a lesser extent, of agonists like 2-thiazolylethylamine, 2-pyridylethylamine, or betahistine. In the case of 2-methylhistamine or 2-(2-aminoethyl)imidazo-1,2-*a*-pyridine (SK&F 71473), the effect was hardly detectable. In most cases the shift was accompanied by a shallowing of the inhibition curve, as expressed by the changes in n_H indexes (Table 2). In contrast, the inhibition curves of antagonists like *d*-chlorpheniramine or mianserin were not significantly modified regarding either IC_{50} values or n_H indexes (Table 2).

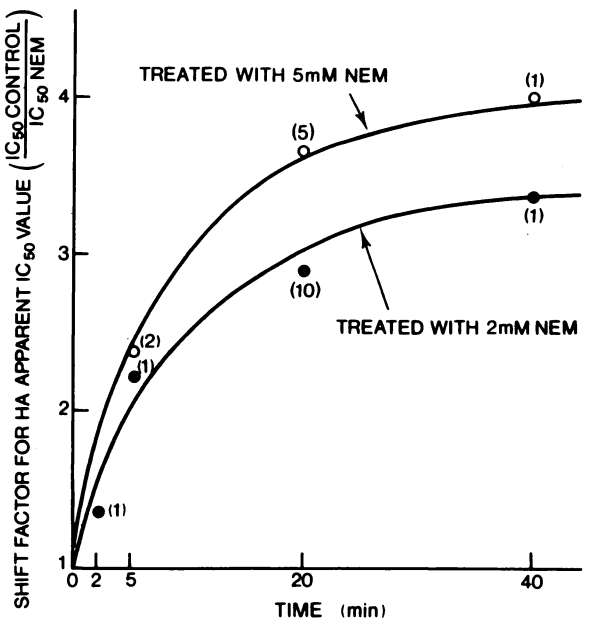

FIG. 4. *Time course of the shift effect produced by NEM on the mean IC_{50} value of HA*

Membranes including paired controls were allowed to react with 2 or 5 mM NEM for various durations up to 40 min. For each condition, the mean IC_{50} value of HA was determined by analysis with a single-component model of the inhibition curve of specific [^{3}H]mepyramine binding. Mean IC_{50} values for control membranes were compared to those of treated ones and the ratios taken as the "shift factors." The number of experiments is indicated in parentheses. Approximate $t_{1/2}$ values were 6 min for 5 mM NEM and 7 min for 2 mM NEM (graphic estimation).

The 2-thiazolylethylamine inhibition curve in membranes treated with 5 mM NEM could be resolved into two components with IC_{50} values of 13 ± 5 μM and 230 ± 116 μM, respectively, each component representing about 50% of the total.

Effects of treatment by other agents modifying thiol groups. At a concentration of 1 mM, iodoacetamide and iodoacetic acid failed to affect the total number of binding sites and the IC_{50} value of HA (Table 3). At 10 mM, they did not modify the inhibition of 50 or 100 μM HA (data not shown). The pseudo Hill index for the inhibition curve of HA was not different from unit. In contrast with NEM, mersalyl and *p*-chloromercuriphenylsulfonic acid markedly inhibited the [^{3}H]mepyramine binding (Table 3). For both these agents, this inhibition was nearly complete in the absence of a washing step and partially reversed by including a washing step or by

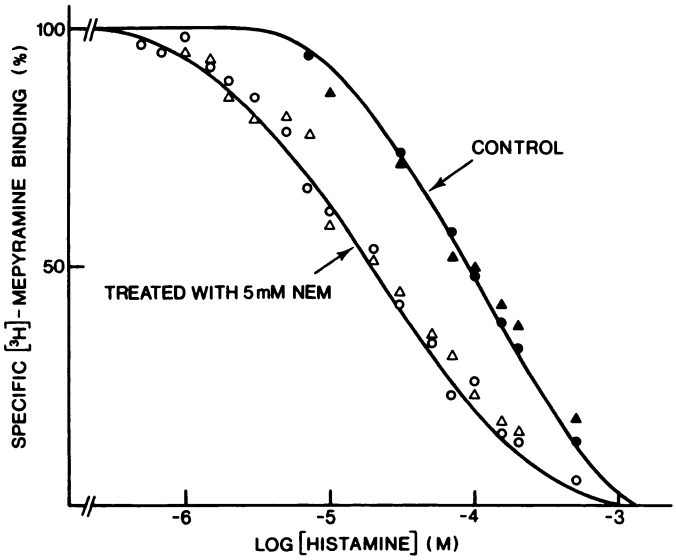

FIG. 5. *Effects of NEM treatment performed in the presence of d-chlorpheniramine, an H_1-receptor antagonist*

Membranes were preincubated at 25° for 25 min with (▲, △) or without (●, ○) *d*-chlorpheniramine (25 nM, final concentration). Each group of membranes was subsequently incubated for 20 min with or without 5 mM NEM and, after addition of 2-mercaptoethanol (10 mM, final concentration) centrifuged and resuspended in buffer for binding assays. In this typical experiment, each value was a single determination. The binding parameters were: for control membranes (●), IC_{50} = 93 μM and the number of specific binding sites 85 fmol/mg protein; when preincubated with *d*-chlorpheniramine (▲), IC_{50} = 93 μM and the number of specific binding sites 93 fmol/mg protein; for NEM-treated membranes (○) IC_{50} = 21 μM and the number of specific binding sites 93 fmol/mg protein; and when preincubated with *d*-chlorpheniramine (△), IC_{50} = 23 μM and the number of specific binding sites 89 fmol/mg protein.

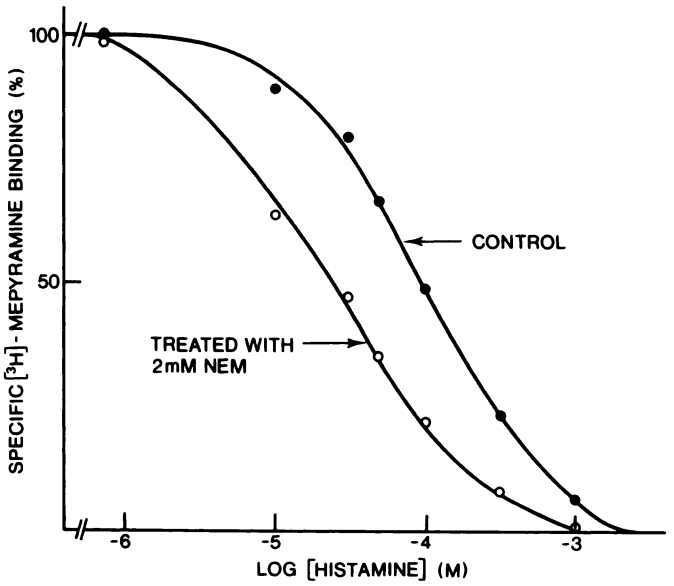

FIG. 6. *Effects of NEM treatment on the inhibition by HA of [^{3}H] mepyramine binding to solubilized H_1-receptors*

Digitonin-solubilized receptors were treated for 20 min with 2 mM NEM and the reaction stopped by addition of 4 mM 2-mercaptoethanol. The binding assays were performed in the presence of 0.6 nM [^{3}H] mepyramine as described under "Experimental Procedures." Each *point* represents the mean from 2 separate experiments (triplicate determinations). The specific binding was 78 ± 8 fmol/mg protein for control and 45 ± 6 fmol/mg protein for the NEM-treated solubilized membranes. The inhibition parameters for HA as determined by a one-component analysis were: IC_{50} = 99 ± 8 μM, n_H = 1.04 ± 0.07 (controls), IC_{50} = 23 ± 4 μM, n_H = 0.77 ± 0.08 (NEM treated).

addition of 2-mercaptoethanol (not shown). Preliminary experiments suggest that mianserin (at a concentration corresponding to 5-fold its K_i value) exerts some protection against this inhibition. The IC_{50} value of HA, tentatively estimated on the binding sites present after a washing step following the treatment with these two agents, was found to be slightly decreased as compared to the control value (data not shown).

DISCUSSION

The present data show that treatment of cerebellar membranes with NEM does not modify the binding of antagonists but significantly increases the affinity of HA and agonists at H_1-receptors. The lack of modification of antagonist binding is clearly shown by the unchanged K_d and B_{max} values of [^{3}H]mepyramine (Fig. 1) as well as by the unmodified affinity of H_1-receptor blockers like *d*-chlorpheniramine or mianserin (Table 2). In contrast, the organic mercurials *p*-chloromercuriphenylsulfonic acid and mersalyl, two other thiol group reagents, drastically reduced [^{3}H]mepyramine binding (Table 3). A similar difference between the effects of NEM and organic mercurials regarding antagonist binding was observed in the case of dopamine (15), opiate (19), and muscarinic cholinergic (13) receptors and explained by a steric inhibition of binding occurring in the case of the bulky organic mercurials but not in that of the much smaller ethylmaleimide residue.

NEM treatments of membranes significantly increased the affinity of HA as shown by the shift in mean IC_{50} values. The effect was time (Fig. 4) and concentration dependent (Table 2) and was apparently irreversible since it persisted after washing out the excess of reagent from membranes.

In NEM-treated membranes, the concentration-inhibition curve of 2-thiazolylethylamine, a full agonist at H_1 receptor from various tissues (1, 3, 18) was shifted to an extent only slightly lower than that of HA (Table 2). In contrast, the affinity of 2-(2-aminoethyl)imidazo-1,2-*a*-pyridine (SK&F 71473), a compound with an intrinsic activity of half that of HA on the guinea pig ileum (18) although this preparation responds with a large receptor "reserve," was not affected in membranes treated with 5 mM NEM (Table 2). Hence, this compound behaved as an antagonist. For other partial agonists like betahistine (3, 20) the shift factor was intermediate (Table 2) suggesting that the effect of NEM is significant for full agonists only.

The lack of effect of two thiol reagents, iodoacetic acid and iodoacetamide (Table 3), does not rule out that a thiol group is the site of action of NEM. Although NEM may react with imidazole and α-amino groups (12) this is very unlikely to occur under the mild conditions presently used (neutral pH and millimolar reagent concentrations). Furthermore, a similar discrepancy between

TABLE 2

Effects of NEM treatment on the inhibition by H_1-receptor agonists and antagonists of [^{3}H]mepyramine binding to cerebellar membranes

Membranes were treated with 2 or 5 mM NEM and the inhibition of [^{3}H]mepyramine (0.6 nM) binding measured in experiments whose data are shown in Fig. 2 (in the presence of 100 mM Na^+) or in similar experiments. The inhibition parameters were obtained by analysis of data with the one-component model so as to obtain the mean IC_{50} value of each inhibitor. The IC_{50} shift factor was calculated as the ratio of apparent IC_{50} value for control membranes to apparent IC_{50} value for NEM-treated membranes. For 2 mM NEM treatment, each experiment was conducted with 6 different inhibitor concentrations (triplicate determinations). For 5 mM NEM treatment, each experiment was conducted with 10–18 different inhibitor concentrations (single or duplicate determinations). Experiments were replicated 2–5 times except data for 2-pyridylethylamine and 2-methylhistamine which represent a single experiment.

	Control		NEM treated					
			2 mM			5 mM		
	IC_{50} value	n_H	IC_{50} value	IC_{50} shift factor	n_H	IC_{50} value	IC_{50} shift factor	n_H
	μM		*μM*			*μM*		
Agonists								
Histamine	78 ± 3	1.00 ± 0.03	27 ± 4*	2.8	0.77 ± 0.06*	21 ± 2*	3.7	0.69 ± 0.03*
2-Thiazolylethylamine	112 ± 10	0.98 ± 0.07	50 ± 5*	2.2	0.89 ± 0.06	49 ± 7*	2.3	0.70 ± 0.04*
Betahistine	101 ± 5	1.11 ± 0.08	73 ± 6*	1.5	1.08 ± 0.05	63 ± 4*	1.7	0.97 ± 0.06
2-Pyridylethylamine	172 ± 11	0.94 ± 0.04	93 ± 12*	1.8	0.85 ± 0.08			
2-Phenylhistamine (SK&F 71491)	11 ± 1	1.22 ± 0.06				7 ± 1	1.6	1.02 ± 0.06
2-Methylhistamine	226 ± 22	1.16 ± 0.13				198 ± 21	1.4	1.03 ± 0.08
2-(2-Aminoethyl)imidazo-1,2-*a*-pyridine (SK&F) 71473)	16 ± 1	1.23 ± 0.07				15 ± 1	1.1	1.10 ± 0.02
	nM		*nM*					
Antagonists								
d-Chlorpheniramine	6.8 ± 1.4	0.86 ± 0.14	5.0 ± 1.8	1.4	0.88 ± 0.25			
Mianserin	9.7 ± 1.4	1.15 ± 0.07	9.7 ± 2.5	1.0	1.12 ± 0.26			

*$p < 0.05$.

TABLE 3

Effect of various sulfhydryl-modifying reagents on [^{3}H]mepyramine binding

The incubation time was 60 min for iodoacetamide and iodoacetic acid and 20 min for *p*-chloromercuriphenylsulfonic acid and mersalyl. For iodoacetamide and iodoacetic acid, the reaction was stopped by addition of 2-mercaptoethanol (2 mM) followed by addition of cold buffer and centrifugation. For the two other reagents, 2-mercaptoethanol was omitted. In all cases a washing step was performed before binding studies. Each agent was tested in two to four experiments. [^{3}H]Mepyramine was used at 0.6 nM.

Treatment	Specific binding of [^{3}H]mepyramine
	fmol/mg protein
None	115
Iodoacetamide (1 mM)	108
Iodoacetic acid (1 mM)	120
p-Chloromercuriphenyl sulfonic acid (1 mM)	28
Mersalyl (0.1 mM)	30

iodoacetamide and NEM effects was also reported for dopamine (15) or opiate receptors (19) and attributed to a participation of the microenvironment of the thiol in the accessibility or reactivity of the latter to various alkylating agents (12). It is obvious, however, that the actual nature of the group modified by thiol reagents in the H_1-receptor awaits detailed chemical analysis of the reaction products. The putative thiol alkylated by NEM seems located outside the binding domain of either agonists or antagonists since these agents, tested at concentrations representing 25–150 times their dissociation constants, did not significantly protect the H_1-receptor (Fig. 5 and see "Results"). Under similar conditions, agonists and antagonists protect opiate receptors against the NEM effects (19). In addition the NEM effects on β-adrenergic and muscarinic receptors are lost upon solubilization (14, 21) in agreement with other observations suggesting that critical thiol groups are located not on the ligand recognition unit but on the guanyl nucleotide regulatory proteins (21–23). In contrast, HA affinity was still increased after NEM treatments performed either on solubilized receptors (Fig. 6) or on membranes thereafter submitted to solubilization (not shown), suggesting that the putative thiol is located on the H_1-receptor itself and not on an easily dissociable regulatory protein. Indeed, H_1-receptors, although participating in a complex manner in the cyclic AMP response to HA (2, 3) are not coupled with an adenylate cyclase unit, and guanyl nucleotides elicit only a 2-fold decrease in HA potency (24).

How can the NEM-induced change in agonist affinity be interpreted? It is important to notice that the leftward shift in concentration-inhibition curves of these compounds was always accompanied by a shallowing of these curves, as expressed by the decreases in pseudo Hill coefficient (n_H). Furthermore, the shift factor in IC_{50} values of the various agents was directly related with the change in pseudo Hill coefficients (Table 2). Computer analysis of these data using a two-component model supports the idea that NEM induced a heterogeneity among [^{3}H]mepyramine-binding sites. Thus, part of them (24% after 2 mM NEM and 48% after 5 mM NEM) displayed a 30-fold increase in affinity for HA (Table 1). This view is consistent with the observation that the IC_{50} value of HA for the low affinity component in NEM-treated membranes was similar to that of the single component in control membranes.

Only about 50% of receptors was converted into a high affinity state for HA, even after treatment with 5 mM

NEM (note, however, that the effect was not complete after a 20-min treatment as shown in Fig. 4). This suggests that H_1 receptors might be heterogeneous in control membranes, part of them being insensitive to alkylation, and recalls findings on β-adrenergic receptors among which a NEM-resistant subpopulation is not functionally coupled to the adenylate cyclase system (22).

That NEM treatment stabilizes various receptors in a particular conformation distinct from that of the activated state is well established, although this may result in either a decrease (opiate receptors) (19) or an increase of agonist affinity (muscarinic receptors) (13, 25). The unmodified [^{3}H]mepyramine-binding sites with low affinity for HA could represent the functional H_1-receptors in view of the similarity between the IC_{50} value of the amine and its EC_{50} value to trigger responses in cyclic AMP-generating systems with low "receptor reserve" (2, 3). Even on a system with a large "receptor reserve" like the glycogenolytic system in brain slices, the EC_{50} value of HA was slightly higher than its IC_{50} value for the high affinity component in NEM-treated membranes (1). However, such comparisons should be cautious because of the role of the ionic composition of media on H_1-receptor binding. In addition NEM treatments were found to decrease the HA-induced contractions of rabbit aorta, an H_1-receptor-mediated response (11). It is unlikely that the subpopulation of sites with high affinity for HA corresponds to sites labeled with [^{3}H]HA in cerebral membranes because the latter display a much lower affinity for mepyramine and may correspond to a conformational state of the H_2-receptor (26).

In analogy with other receptor systems (21–23, 27, 28) it might be hypothesized that NEM stabilizes the H_1-receptor in one of the conformational states between which it fluctuates under the action of agonists. Desensitization of cerebral H_1-receptors exposed to HA and agonists has been described (8, 29). This process was accompanied by a limited although significant decrease in the number of [^{3}H]mepyramine-binding sites, together with a tendency to an increase in HA affinity for these sites, and it was proposed that desensitization corresponds to a transition of the H_1-receptor into a high affinity state in which it would more tightly bind HA or agonists (8). Such a mechanism seems associated with the desensitization of nicotinic receptors (30), but it is as yet premature to conclude that the NEM-induced state is similar to the desensitized state of the H_1-receptor.

Sodium ions are known to induce a decrease in the affinity of agonists at H_1-receptors (24) as well as at opiate receptors. For the latters, Na^+ exerted specific protection against inactivation of [^{3}H]naltrexone binding by NEM (16). At H_1-receptors, the effect of NEM is similar in the presence or in the absence of 100 mM Na^+ (Fig. 2) and, therefore, there is no evidence that the configurational changes of the receptor induced by Na^+ ions are related to that induced by alkylation of sulfhydryl groups. Finally, the possibility of unmasking another NEM-sensitive group upon solubilization is raised by the decrease in the number of binding sites in the solubilized membranes treated by NEM. In conclusion, as in the case of other receptors, NEM treatment induces selective changes in the recognition of HA and agonists by the H_1-receptor, but they are not yet easily interpretable, namely because of the limited knowledge of the nature of molecular events involved in the coupling of this receptor activation to final biological responses.

ACKNOWLEDGMENT

We wish to thank C. R. Ganellin (Smith Kline and French Laboratories, England) for the expert choice and generous gift of various histamine agonists used in this study.

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Send reprint requests to: Dr. Jean-Charles Schwartz, Unité 109 de Neurobiologie, Centre Paul Broca de l'INSERM, 2 ter rue d'Alésia, 75014, Paris, France.