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Selective Effects of an Essential Sulfhydryl Group on the Activation of Dopamine- and Guanine Nucleotide-Sensitive Adenylate Cyclase

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SUMMARY

Treatment of membranes from the dog caudate nucleus with sulfhydryl alkylating agents, *N*-ethylmaleimide and *p*-chloromercuribenzoate, results in selective inhibition of dopamine-sensitive adenylate cyclase activity that can be distinguished from effects on basal enzyme activity. Fifty per cent inhibition of dopamine-sensitive adenylate cyclase activity was observed in the presence of 10^{-5} M *N*-ethylmaleimide and 3×10^{-6} M *p*-chloromercuribenzoate. *N*-Ethylmaleimide (10^{-5} M or less) also inhibited GTP- and NaF-stimulated adenylate cyclase activity, but had no effect on basal adenylate cyclase activity (assayed in the presence of magnesium) and on enzyme activity assayed in the presence of manganese. The reducing agents dithiothreitol, 2-mercaptoethanol, glutathione, and cysteine had no inhibitory effect on dopamine-sensitive adenylate cyclase activity. Pretreatment of membranes with guanyl-5′-yl imidodiphosphate or guanosine 5′-*O*-(3-thio)triphosphate prior to incubation with *N*-ethylmaleimide prevented the inhibitory effect of *N*-ethylmaleimide on adenylate cyclase activity. The results suggest that a reactive sulfhydryl group in the domain of the GTP-binding protein is important for the coupling of the components of the dopamine-sensitive adenylate cyclase complex in brain.

INTRODUCTION

Considerable evidence suggests that the receptor-coupled adenylate cyclase system is composed of several distinct components: (a) the catalytic unit that converts ATP to cyclic AMP, (b) a guanine nucleotide regulatory protein, and (c) a receptor-binding site for the hormone or neurotransmitter (1). Recent studies on the interactions between these components in the plasma membrane suggest that hormones or neurotransmitters, guanine nucleotides, and sodium fluoride promote the assembly of the active enzyme. The participation of reactive sulfhydryl groups in the assembly of these components and in the activation of adenylate cyclase has been proposed by several investigators from studies with *N*-ethylmaleimide and iodoacetate, agents that form irreversible complexes with sulfhydryl groups (2–5).

In previous studies we explored the role of sulfhydryl groups in the regulation of dopamine receptor binding (6). We examined the effects of thiol reducing agents, such as dithiothreitol, and alkylating agents, such as *N*-ethylmaleimide, on receptor affinity and number. The results showed that dithiothreitol decreased the specific binding of the agonist ligand, [^{3}H]dopamine, to striatal membranes without changing the binding of the antagonist ligand, [^{3}H]spiroperidol (6). Similar results were observed with *N*-ethylmaleimide. The inhibition of agonist binding produced by reducing agents appeared to be specific for the high-affinity form of the receptor and affected only part of the dopamine receptor population. This inhibition was reversed by the addition of hydrogen peroxide. These data suggest that conformational changes in the dopamine receptor may be influenced by its oxidation-reduction state and that sulfhydryl reagents may be useful for distinguishing between different subpopulations of dopaminergic binding sites. These results are similar to the effect of sulfhydryl agents on the binding of other hormones to receptors (2).

Several studies on the *beta*-adrenergic receptor indicate that reactive sulfhydryl groups regulate the interaction between the receptor and adenylate cyclase (3–5). Vauquelin and Maguire (7) reported that the alkylating agent *N*-ethylmaleimide inactivated approximately 50% of the *beta*-adrenergic receptors in turkey erythrocyte membranes. In recent studies they and Lucas *et al.* (8) showed that the inhibitory effect of *N*-ethylmaleimide on adrenergic receptor binding is specific for agonist ligands and that the effect on binding correlates with the ability of *N*-ethylmaleimide to inhibit adenylate cyclase activity. These results raise the possibility that a reactive sulfhydryl group is associated with a specific subpopulation of receptors that are coupled to the adenylate cyclase complex. They also suggest that reactive sulfhydryl groups may also be associated with the coupling protein.

This study is designed to explore the possibility that a reactive sulfhydryl group is associated with one of the

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components of the dopamine-sensitive adenylate cyclase complex. The system chosen for the study was the adenylate cyclase from dog caudate nucleus membranes, which is stimulated by dopamine and which has many typical features and requirements of hormone-sensitive adenylate cyclase. A consistent pattern of observed effects of sulfhydryl agents on adenylate cyclase activity led to the conclusion that the major target of these agents at low concentrations is the guanine nucleotide regulatory protein.

EXPERIMENTAL PROCEDURES

Animals. Mongrel dogs were kept for at least 2 months under controlled conditions of light and temperature and provided a diet which consisted of fresh water ad libitum and dog food twice daily. The dogs were killed by pentobarbital overdose. The caudate nucleus was excised immediately, frozen on dry ice, and stored at −80°.

Adenylate cyclase assay. Frozen caudate from dog brain was thawed and suspended in 30 volumes of 0.32 M sucrose/2 mM EGTA,[1] pH 7.4. The homogenate was centrifuged at 50,000 × *g* for 20 min and the resulting pellet was washed twice with the same buffer. This fraction was resuspended in the original volume of 2 mM Tris maleate/2 mM EGTA at pH 7.4. The washed membrane fraction for the adenylate cyclase assay was always prepared fresh on the same day on which the assay was performed. The standard assay system (final volume 0.5 ml) contained (millimolar concentrations) Tris maleate, 80.2; ATP, 0.3; $MgSO_4$, 2.0; theophylline, 10.0; and EGTA, 0.6. Washed membranes (0.05 ml) were added, plus test substances as indicated. In order to stimulate adenylate cyclase activity with dopamine in washed membranes, it was necessary to include GTP (10^{-5} M) in the assay buffer (9). Membranes were pretreated with *N*-ethylmaleimide or *p*-chloromercuribenzoate for 20 min at 4° in the assay buffer; ATP then was added and the samples were incubated for 2.5 min at 30°. The reaction was terminated by boiling, and cyclic AMP was measured as described previously (9). Protein concentrations were determined as described by the method of Lowry *et al.* (10), using bovine serum albumin as a standard.

Materials. Tris-HCl, EGTA, 3-hydroxytyramine (dopamine), maleic acid, *N*-ethylmaleimide, *p*-chloromercuribenzoate, magnesium sulfate, Gpp(NH)p, and sodium fluoride were purchased from Sigma Chemical Company (St. Louis, Mo.). Dithiothreitol was obtained from Aldrich Chemical Company (Milwaukee, Wis.). GTPγS was a gift from Dr. L. Birnbaumer, Department of Cell Biology, Baylor College of Medicine. GTP was purchased from Boehringer Mannheim Biochemicals (Mannheim, West Germany). Haloperidol was obtained from Janssen Pharmaceuticals (Beerse, Belgium). All other reagents and solvents were of analytical grade or better.

RESULTS

A series of experiments was performed to test the effect of sulfhydryl reagents on dopamine-, fluoride- and guanine nucleotide-stimulated adenylate cyclase activity. Figure 1 shows the effect of *N*-ethylmaleimide pretreatment on basal and dopamine-sensitive adenylate cyclase activity in caudate membranes. In these studies, *N*-ethylmaleimide and dopamine were preincubated with striatal homogenates for 20 min on ice. Following preincubation, the membranes were assayed for adenylate cyclase activity in the presence of *N*-ethylmaleimide as described under Experimental Procedures. *N*-Ethylmaleimide at concentrations up to 5×10^{-5} M decreased dopamine-sensitive adenylate cyclase activity in caudate homogenates without significantly changing basal activity. A 40% inhibition of dopamine-stimulated adenylate cyclase activity was observed in the presence of 10^{-5} M *N*-ethylmaleimide (Fig. 1). Essentially, complete inhibition of adenylate cyclase occurred in the presence of 10^{-3} M *N*-ethylmaleimide. The effect of *p*-chloromercuribenzoate on dopamine-sensitive adenylate cyclase activity in striatal homogenates is also shown in Fig. 1. The results reveal that *p*-chloromercuribenzoate was more potent than *N*-ethylmaleimide as an inhibitor of dopamine-sensitive adenylate cyclase activity. More than 80% of dopamine-stimulated enzyme activity was inhibited by 10^{-5} M *p*-chloromercuribenzoate. Inhibition of dopamine-sensitive adenylate cyclase activity by *p*-chloromercuribenzoate could be completely reversed by dithiothreitol (data not shown). In both *N*-ethylmaleimide- and *p*-chloromercuribenzoate-treated membranes the basal activity was not affected by 10^{-5} M concentrations of the sulfhydryl agents and only slightly reduced by 10^{-4} M. These assays were performed in the presence of the sulfhydryl reagents. However, we obtained identical results if the *N*-ethylmaleimide-treated membranes were washed prior to performing the adenylate cyclase assay. Under these

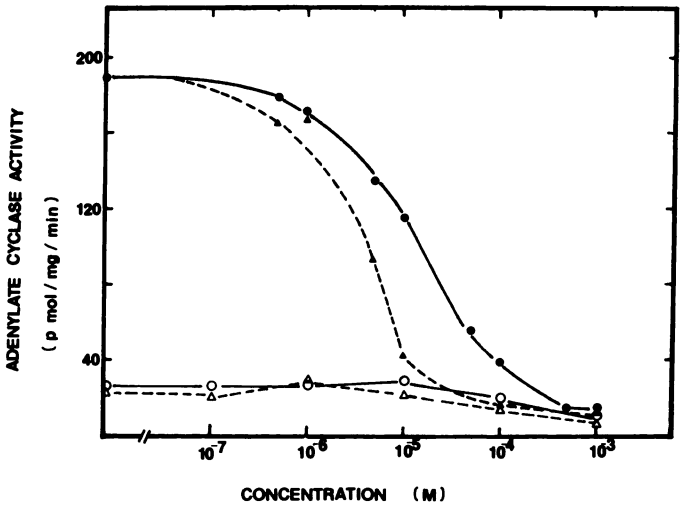

FIG. 1. *Inhibition of adenylate cyclase activity by N-ethylmaleimide and p-chloromercuribenzoate in dog striatal homogenate*

Dog striatal homogenate was prepared as described under Experimental Procedures. Various concentrations of *N*-ethylmaleimide (*circles*) and *p*-chloromercuribenzoate (*triangles*) were added to the homogenate in the presence (●, ▲) and absence (○, △) of 5×10^{-5} M dopamine for 20 min at 4°. The reaction was initiated by adding 0.3 mM ATP to the incubation mixture. Each point represents the mean value of triplicate determinations. The experiment was repeated three times.

[1] The abbreviations used are: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate.

conditions it was necessary to add GTP to the incubation system (9). We also obtained similar results when an ATP-regenerating system was included in the assay buffer and [^{32}P]ATP was used to generate cyclic AMP.

The inhibition of dopamine-sensitive adenylate cyclase by 10^{-5} M *N*-ethylmaleimide was studied as a function of preincubation time to determine whether the alkylating agent had any effect on the rate at which dopamine reaches equilibrium. The data in Fig. 2 indicate that dopamine stimulation of adenylate cyclase reached equilibrium within 10 min at 4° and that this effect was blocked by *N*-ethylmaleimide. The thiol reducing agents glutathione, cysteine, 2-mercaptoethanol, and dithiothreitol were also tested in our system. Whereas one of these agents, dithiothreitol, has been shown to reduce agonist binding in dog striatal membranes (6), these agents had no significant effect on basal or dopamine-stimulated adenylate cyclase activity.

To investigate the possibility that the guanine nucleotide regulatory protein or the catalytic unit was involved in the uncoupling of the dopamine receptor-cyclase complex which is produced by *N*-ethylmaleimide, subsequent experiments were performed on washed caudate membranes which show a GTP requirement for dopamine stimulation of enzyme activity (9). Table 1 shows the effect of pretreatment of caudate membranes with *N*-ethylmaleimide on dopamine- and GTP-sensitive adenylate cyclase. GTP increased adenylate cyclase activity from 153 pmoles/mg of protein per minute to 208 pmoles/mg of protein per minute; this value was reduced to 182 pmoles/mg of protein per minute by *N*-ethylmaleimide treatment. In the presence of 5×10^{-5} M dopamine and 10^{-5} M GTP, adenylate cyclase activity was reduced from 476 pmoles/mg of protein per minute in the absence of *N*-ethylmaleimide to 300 pmoles/mg of protein per minute, a decrease of 37% in the presence of *N*-ethylmaleimide, indicating a possible disruption of coupling between receptor and regulatory protein.

It is noteworthy that basal adenylate cyclase activity (in the presence of magnesium) was not inhibited by *N*-ethylmaleimide (10^{-5} M or less) under conditions which blocked dopamine stimulation of enzyme activity (Figs.

TABLE 1

Effect of N-ethylmaleimide (NEM) on GTP-sensitive adenylate cyclase activity

Dog striatal membranes were pretreated with 5×10^{-5} M *N*-ethylmaleimide for 20 min at 4°, and various concentrations of dopamine with 10^{-5} M GTP were added to initiate the reaction as described under Experimental Procedures. Basal activities (without GTP) in the presence and absence of *N*-ethylmaleimide were 153 ± 91 and 158 ± 14 pmoles/mg/min, respectively.

Addition	Adenylate cyclase activity	
	− NEM	+ NEM
	pmoles/mg/min	
GTP	208 ± 4	182 ± 9
GTP + dopamine (20 μM)	409 ± 28	275 ± 21
GTP + dopamine (50 μM)	476 ± 10	300 ± 11

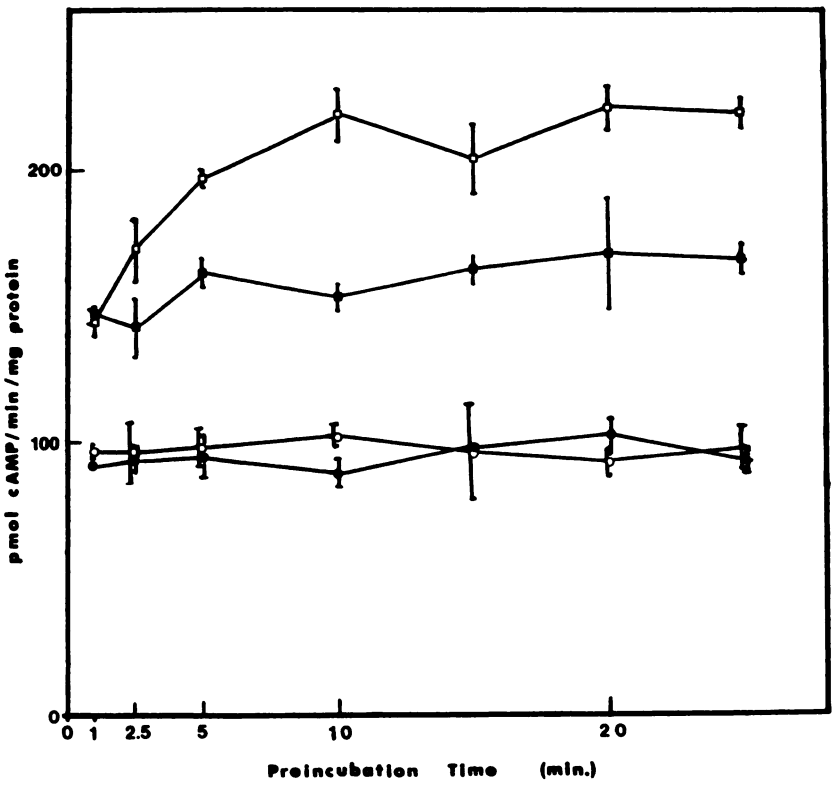

FIG. 2. *Time course for N-ethylmaleimide treatment of membranes and its effect on adenylate cyclase activation by dopamine*

Preincubation at 4° was initiated by the addition of dog caudate homogenate to the incubation medium containing 10^{-5} M *N*-ethylmaleimide (●), 10^{-4} M dopamine (□), or 10^{-5} M *N*-ethylmaleimide plus 10^{-4} M dopamine (■) or none of the above (○) for the time period shown. The reaction was initiated by the addition of 0.3 mM ATP (final concentration) and incubated at 30° for 2.5 min; the reaction was stopped by boiling for 3 min. The mixture was then centrifuged to remove insoluble materials. The amount of cyclic AMP in the supernatant was determined as described under Experimental Procedures.

3 and 4). The results in Table 2 show the effect of *N*-ethylmaleimide treatment on the adenylate cyclase activity measured in the presence of magnesium or manganese. Washed membranes were pretreated with *N*-ethylmaleimide for 20 min at 4°, washed again to eliminate the excess *N*-ethylmaleimide, and then assayed in the presence of magnesium or manganese chloride. The results in Table 2 show that manganese produced well over a 20-fold increase above basal activity (without dopamine or GTP). Catalytic activity was not altered by pretreatment with *N*-ethylmaleimide (5×10^{-5} M or less). However, this concentration of *N*-ethylmaleimide caused more than 50% inhibition (from 372 to 173 pmoles/mg of protein per minute) of dopamine- and GTP-sensitive adenylate cyclase. These data suggest that basal activity and possibly the catalytic unit of adenylate cyclase were not selectively affected by low concentrations of *N*-ethylmaleimide under these assay conditions.

To examine further the effect of *N*-ethylmaleimide on the guanine nucleotide regulatory protein, we measured the enzyme activity following pretreatment with the guanine nucleotide analogues Gpp(NH)p and GTPγS. Pretreatment of nucleotide analogues was carried out in the presence of 5 mM $MgSO_4$ at 37° for 10 min. Recent studies by Iyengar and Birnbaumer (11) have indicated that under these conditions the activation of the enzyme by the guanine nucleotide analogue is optimal as compared with our standard assay procedure. The adenylate cyclase assay was slightly modified to include 1 mM ATP and 5 mM $MgSO_4$ in order to maximize the effect of the guanine nucleotide analogue. The results in Fig. 3 show that the enzyme activity in membranes that were treated with the guanine nucleotide analogue prior to *N*-ethylmaleimide was essentially identical with that measured in the presence of the nucleotide alone. However, pretreatment with *N*-ethylmaleimide for 20 min at 4° prior to exposure to Gpp(NH)p or GTPγS resulted in more than a 50% decrease in enzyme activity. Pretreatment with *N*-ethylmaleimide for 1 hr at 4° prior to exposure to the guanine nucleotide analogue resulted in enzyme activity comparable to basal levels (data not shown). It appears that the inhibition of sodium fluoride stimulation of adenylate cyclase produced by *N*-ethylmaleimide follows a pattern similar to that for guanine nucleotides (Fig. 4). These data strongly suggest that the alkylating compound *N*-ethylmaleimide reacts with a sulfhydryl group on or near the nucleotide binding site which is essential in the coupling mechanism associated with receptor function. However, they do not rule out the possibility that a reactive sulfhydryl group on the receptor itself could be mediating this effect, as has been suggested for the *beta*-receptor (3, 12).

TABLE 2

Effect of N-ethylmaleimide (NEM) on dopamine-, GTP-, and manganese-sensitive adenylate cyclase

Dog striatal membranes were treated with *N*-ethylmaleimide for 20 min and washed two times with 20 mM Tris-HCl (pH 7.4) before assay. Basal activity represents the activity measured in the standard procedure with $MgSO_4$ (2 mM). The concentrations of dopamine, GTP, and NaF were 10^{-4} M, 10^{-5} M, and 10^{-2} M, respectively. $MnCl_2$ (2 mM) was substituted for $MgSO_4$ in manganese-stimulated activity.

Condition	Adenylate cyclase activity		
	No treatment	+NEM, 25 μM	+NEM, 50 μM
	pmoles/mg/min		
Basal	93 ± 21	93 ± 9	80 ± 24
Dopamine + GTP	372 ± 118	333 ± 41	173 ± 12
Manganese	2381 ± 133	2713 ± 240	2647 ± 85

An examination of the effect of antagonist pretreatment on the *N*-ethylmaleimide-induced decrease in dopamine-sensitive adenylate cyclase provides some information about the involvement of the receptor in the inactivation process. Figure 5 shows the effect of haloperidol (10^{-6} M) pretreatment on the inhibition of dopamine-sensitive adenylate cyclase by *N*-ethylmaleimide. The concentration of haloperidol used was sufficient to inhibit dopamine-sensitive adenylate cyclase activity completely (Table 3). In the experiment, haloperidol was added to striatal membranes at 4° 30 min prior to the addition of *N*-ethylmaleimide. The membranes were then centrifuged and washed twice, and adenylate cyclase activity was measured in the presence and absence of dopamine. The data reveal that pretreatment with haloperidol alone (Fraction *c*) did not affect the ability of dopamine to stimulate adenylate cyclase activity; in fact, the enzyme activity was somewhat enhanced as compared with control. Membranes treated with haloperidol followed by the addition of *N*-ethylmaleimide did not protect against the inhibitory action of the sulfhydryl agent of dopamine stimulation of adenylate cyclase activity. Pretreatment with receptor agonists also did not protect against inactivation by alkylating agents (data not shown). According to the above observations, receptor occupancy does not alter the location of the sensitive sites which undergo reductive alkylation.

CONCLUSION

The regulatory mechanism of hormone- or neurotransmitter-sensitive adenylate cyclase stimulation has been under extensive investigation for the last decade. These studies have identified three components of the cyclase complex: receptor, regulatory protein, and the catalytic unit. The activity of the receptor and the adenylate cyclase has been reported to be reduced by compounds that bind to thiol groups (4, 7). The results reported here clearly support a role for thiol groups in the coupling of the dopamine receptor to the regulatory protein-adenylate cyclase complex. In this study we found that pretreatment of caudate membranes with low concentrations of *N*-ethylmaleimide (10^{-5} M or less) reduced dopamine stimulation of adenylate cyclase activity. Under these conditions basal enzyme activity (in the presence of magnesium) and manganese-sensitive enzyme activity were not inhibited.

Early studies on the effects of sulfhydryl reagents on basal and fluoride-sensitive adenylate cyclase activity led to the suggestion that sensitive thiol groups are associated with the catalytic subunit of adenylate cyclase. For example, studies on the soluble adenylate cyclase from *Streptococcus salivarius*, which was partially purified

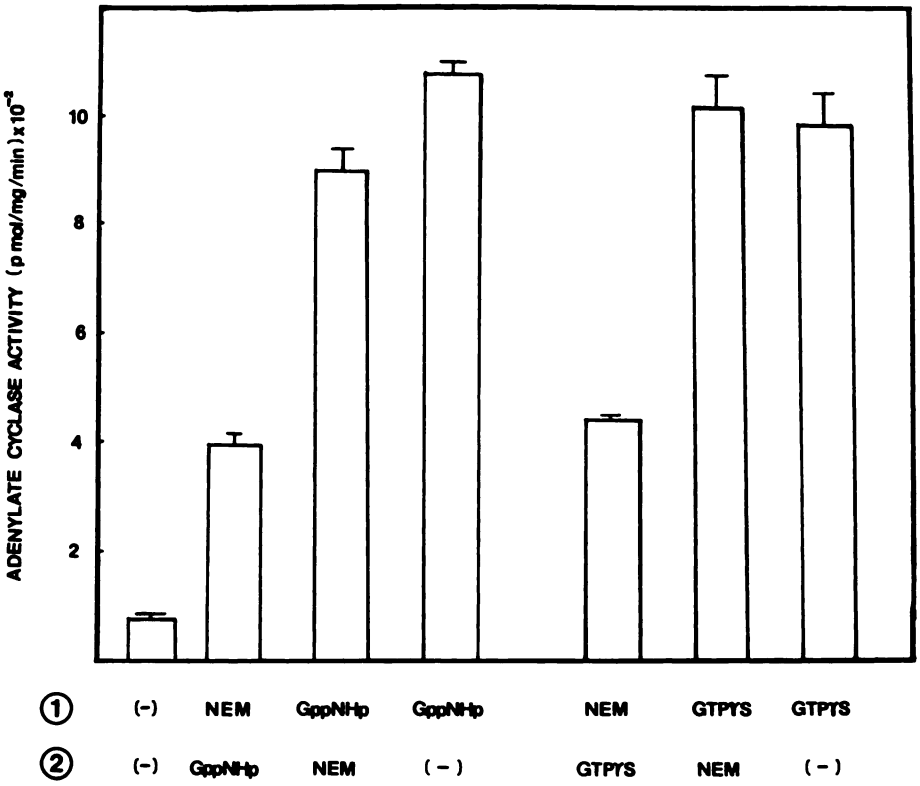

FIG. 3. *Adenylate cyclase activity after pretreatment with combinations of N-ethylmaleimide and guanine nucleotide analogs*

Pretreatment of membranes was carried out in 20 mM Tris-HCl/5 mM $MgSO_4$ buffer (pH 7.4) with 6×10^{-5} M *N*-ethylmaleimide (*NEM*) for 20 min at 4° and Gpp(NH)p (10^{-5} M) or GTPγS (5×10^{6} M) for 10 min at 37°. Following each treatment the membranes were washed twice with the same buffer. The enzyme activity was determined under the standard conditions except for the presence of 1 mM ATP and 5 mM $MgSO_4$.

(13), and adenylate cyclase from lymphoma S49 cells (14) showed that enzyme activity was reduced by sulfhydryl reagents. In these studies, which appear at variance with the data we report here, higher concentrations of sulfhydryl reagents were used (millimolar). We also found that millimolar concentrations of *N*-ethylmaleimide inhibited basal adenylate cyclase activity (in the presence of magnesium). This raises the possibility that the catalytic unit is not as sensitive to sulfhydryl group modification as is the receptor or coupling protein. Moreover, it has been shown that high concentrations of *N*-ethylmaleimide can bind nonspecifically to imidazole groups of histidine residues (15). Thus, the suggestion that the inhibitory effect of these agents reflects sulfhydryl group involvement in catalytic activity must be viewed with caution.

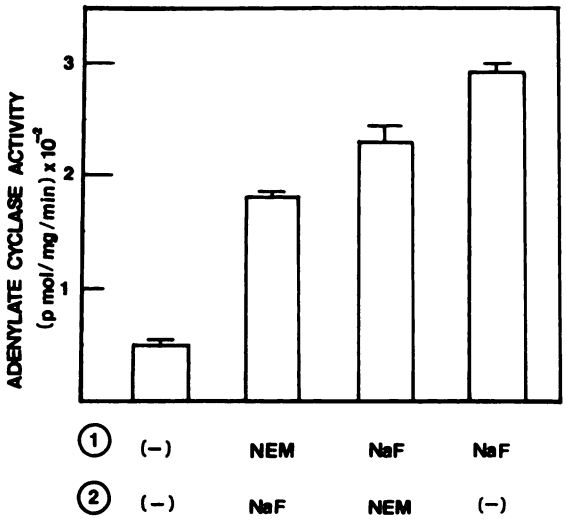

FIG. 4. *Adenylate cyclase activity after pretreatment of membranes with N-ethylmaleimide and sodium fluoride*

Pretreatment of membranes with 5×10^{-5} M *N*-ethylmaleimide (*NEM*) or 10^{-2} M NaF for 20 min at 4° was carried out the same way as described in Fig. 5. Following each treatment the membranes were washed twice with 20 mM Tris-HCl (pH 7.4) and assayed as described under Experimental Procedures.

In this study we determined that low concentrations (micromolar) of *N*-ethylmaleimide were useful for evaluating the role of sulfhydryl groups in the coupling between the receptor and nucleotide protein of the cyclase system. The data show that reductive alkylation inhibited the hormone- and guanine nucleotide-sensitive adenylate cyclase activity without altering basal activity. The guanine nucleotide analogues Gpp(NH)p and GTPγS protected against enzyme inactivation by sulfhydryl reagents. These results suggest that *N*-ethylmaleimide competes for the guanine nucleotide binding site on the regulatory protein and that the location of the reactive sulfhydryl group is on this protein. Convincing evidence which supports this conclusion recently has been provided by Korner *et al.* (16). Their data suggest that the guanine nucleotide binding protein is the component which exposes a specific sulfhydryl group upon interaction of the hormone-receptor complex for the *beta*-adre-

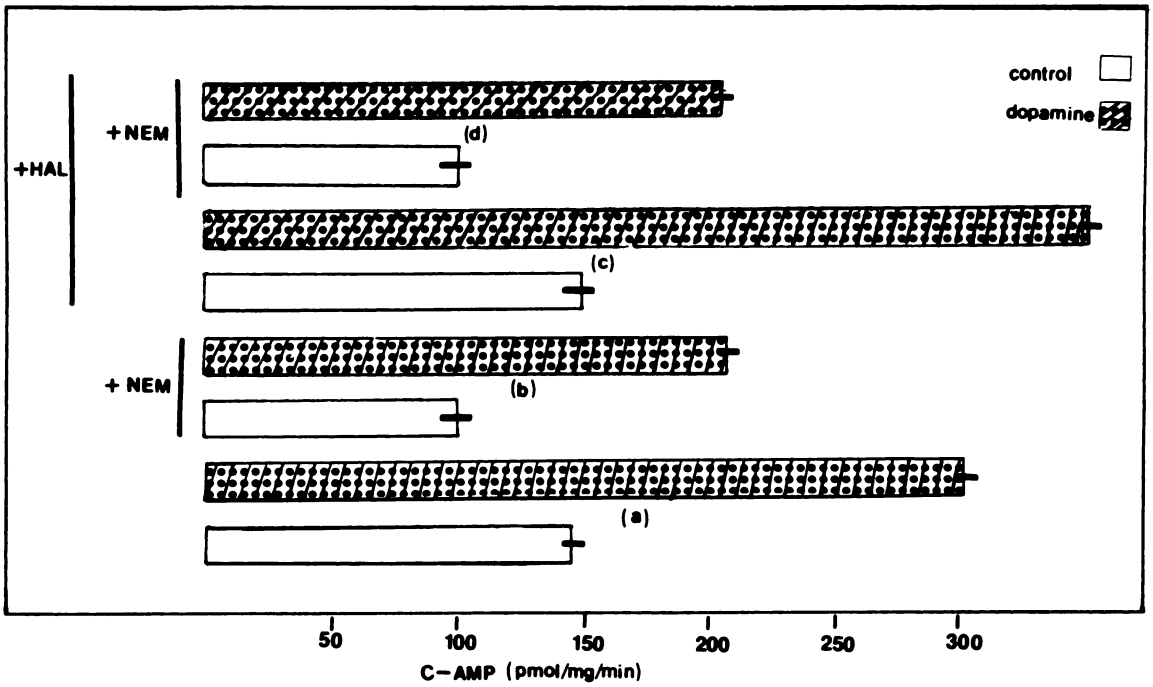

FIG. 5. *Effect of haloperidol on N-ethylmaleimide-induced inhibition of dopamine-sensitive adenylate cyclase*

Dog striatal membranes were prepared as described under Experimental Procedures. The homogenate was divided into four aliquots: Fraction *a* was the control. This homogenate was placed on ice for the duration of the treatment period. Fraction *b* was treated with *N*-ethylmaleimide (*NEM*) for the last 20 min of the treatment period. Fraction *c* was preincubated with haloperidol (*HAL*) (10^{-6} M) for the last 30 min of the treatment period; Fraction *d* was preincubated with haloperidol (10^{-6} M) for the first 30 min of the treatment period; *N*-ethylmaleimide (10^{-5} M) then was added to the membranes for 20 min in combination with the antagonist. At the end of the treatment period for all fractions, the membranes were pelleted, washed twice, and resuspended in buffer. Adenylate cyclase was assayed in the presence and absence of dopamine (100 μM). The data shown are representative of two separate experiments.

nergic system. An alternative explanation which must be considered is the possibility that the binding of the guanine nucleotide analogue at its active site produces a conformational change in the regulatory protein which renders the sulfhydryl group at some *other location* within the domain of the regulatory protein inaccessible to alkylating agents. Clearly distinguishing between these two alternatives awaits the further purification of the components of the cyclase complex and the study of their interaction using a reconstitution protocol.

In summary, the results of this study which demonstrate that pretreatment with *N*-ethylmaleimide selectively inhibits hormone-stimulated adenylate cyclase activity without altering basal enzyme activity, and that pretreatment with guanine nucleotides protects against this inhibition, support a role for a specific sulfhydryl group in hormone receptor-cyclase coupling. The data suggest that the ability of the receptor to be coupled functionally to the cyclase (a) is mediated by a coupling protein which binds guanine nucleotide and (b) involves a reactive sulfhydryl group which is located in the domain of the coupling protein. Studies are in progress to determine whether the reactive sulfhydryl group is chemically involved in the coupling process or whether the alkylation of this group blocks access of the guanine nucleotide to its binding site.

TABLE 3

Effect of haloperidol on dopamine-stimulated adenylate cyclase activity

Data represent means ± standard error of the mean from two separate experiments. Enzyme activity was assayed as described under Experimental Procedures. Haloperidol alone at these concentrations did not significantly change basal activity.

Addition	Adenylate cyclase activity
	pmoles/mg/min
None	94 ± 3
Dopamine (10^{-4} M)	228 ± 13
Dopamine + haloperidol (10^{-8} M)	190 ± 8
Dopamine + haloperidol (10^{-7} M)	164 ± 10
Dopamine + haloperidol (10^{-6} M)	100 ± 4

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