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Multiple Reactive Sulfhydryl Groups Modulate the Function of Adenylate Cyclase Coupled *Beta*-Adrenergic Receptors

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SUMMARY

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The *beta*-adrenergic receptor adenylate cyclase complex in the frog erythrocyte contains at least two reactive sulfhydryl groups, which have been identified by their different sensitivities to N-ethylmaleimide (NEM). Adenylate cyclase catalytic activity is completely inhibited by [NEM] = 1 mM. In contrast, ability of *beta*-adrenergic agonists to form a high affinity guanine nucleotide sensitive state is reduced by NEM only at concentrations ≥ 1 mM. The inhibition of cyclase activity did not affect high affinity agonist binding nor perturb the ability of guanine nucleotides to reduce agonist affinity as determined in direct radioligand binding assays utilizing the *beta*-adrenergic agonist [^{3}H]hydroxybenzylisoproterenol and (-)isoproterenol competition for antagonist [^{3}H]dihydroalprenolol binding. Preincubation of frog erythrocyte membranes with antagonists or guanine nucleotide did not alter the effect of NEM on agonist affinity. However, once formed by preincubation of membranes with agonist, the high affinity state is resistant to the effects of NEM. The data suggest that the sulfhydryl group modulating agonist affinity 1) is located on the receptor complex but distal to the hormonal binding site and 2) may be associated with guanine nucleotide regulation of agonist affinity.

The widely different sensitivities of adenylate cyclase activity and agonist binding affinity toward NEM have been exploited to investigate the mechanism of desensitization in frog erythrocytes. Incubation of intact erythrocytes with NEM under appropriate conditions inactivates the cyclase enzyme without altering the ability of membranes prepared from these cells to bind agonist with high affinity. This NEM treatment prior to prolonged exposure to (-)isoproterenol prevents receptor desensitization in frog erythrocytes, suggesting that agonist occupancy of a nucleotide-sensitive receptor is insufficient, by itself, to initiate the receptor regulation process.

INTRODUCTION

Through the development of radioligands, it has been possible to demonstrate directly that *beta*-adrenergic agonists and antagonists competitively bind to the same population of receptor sites located in the plasma membranes of target tissues (1, 2). The binding of agonists to receptors, however, possesses unique characteristics that are qualitatively different from the binding of antagonists:

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1) Agonist binding initiates the stimulation of the enzyme adenylate cyclase resulting in an increase in the intracellular cAMP concentration (3, 4).

2) The apparent affinity of an agonist for its receptor is decreased in the presence of guanine nucleotides (5, 6).

3) Chronic exposure of receptors to agonists leads to a blunting of the biological response to a subsequent challenge by agonist (7–17). One mechanism of this desensitization appears to be a reduction in the number of functional receptors as determined in radioligand binding experiments (12–17).

Early radioligand binding studies of *beta*-adrenergic receptors were conducted using tritiated (18, 19) or iodinated (20, 21) antagonist compounds. Recently, this laboratory has developed a radioactively labeled agonist, [^{3}H]hydroxybenzylisoproterenol ([^{3}H]HBI)[3], which can be employed to investigate directly agonist binding to receptors (1, 22, 23). In purified membrane preparations from frog erythrocytes, [^{3}H]HBI binding to *beta*-adrenergic receptors is of high affinity and is characterized kinetically by a very slow dissociation rate (1). Upon the addition of guanine nucleotides [^{3}H]HBI is rapidly dissociable from the receptor, thus decreasing the apparent affinity of the agonist ligand. It is the slowly reversible binding of agonist ligands to the *beta*-adrenergic receptor, in the absence of guanine nucleotides, which we shall refer to as agonist induced high affinity state.

[^{3}H]HBI high affinity binding is also modulated by reagents that perturb the coupling of the receptor to adenylate cyclase (1, 23). Treatment of membranes with sulfhydryl reagents, filipin, or EDTA prevents agonist-induced formation of the high affinity state and likewise prevents the ability of agonists to stimulate adenylate cyclase. It has been proposed, therefore, that the formation of the high affinity complex of the agonist with the receptor is related to the unique function of these agents to stimulate adenylate cyclase and to induce desensitization.

In the present study we have employed the sulfhydryl reagent N-ethylmaleimide to probe the relationship of the various functional components of the *beta*-adrenergic receptor-adenylate cyclase system to the formation of the agonist induced high affinity state. These components include the receptor binding moiety, the cyclase catalytic unit, and putative guanine nucleotide coupling component (24–27).

We have also used N-ethylmaleimide in studies with intact frog erythrocytes to focus on the role of agonist high affinity binding in the process of desensitization through the mechanism of receptor regulation.

MATERIALS AND METHODS

Materials. The radioligands employed in the present study were [^{3}H]DHA, 58 Ci/mmol and [^{3}H]HBI 12-17 Ci/mmol. The sources, purity, and biological activity of the radioligands, as well as the other materials used, have been previously reported (18, 22).

Partially purified frog erythrocyte membranes. Washed frog erythrocytes were prepared as previously described (18). The washed cells were lysed by resuspension in cold water for 5 min, followed by homogenization in a Potter-Elvehjem homogenizer for 15 strokes. The lysate was mixed with an equal volume of cold Buffer A (75 mM Tris-HCl, 12.5 mM $MgCl_2$, 1.5 mM EDTA, pH 7.5) and centrifuged 30,000 × *g* for 10 min. The resulting pellet from 2–3 ml packed cells was resuspended in 20 ml of cold Buffer A and layered over a 50% sucrose solution containing 50 mM Tris-HCl and 10 mM $MgCl_2$ (pH = 8.1) and centrifuged at 1,200 × *g* for 10 min at 4°. The sucrose buffer interface was harvested and centrifuged at 30,000 × *g* for 10 min. The pellet was finally resuspended in Buffer A.

Radioligand binding assays. Erythrocyte membranes (0.2–0.4 mg of protein/ml) were incubated in a final volume of 1 ml with [^{3}H]HBI, 5–15 nM, or [^{3}H]DHA 2–3 nM for 10 min at 37° in Buffer A. Catechol (0.44 mM) and ascorbic acid (1.1 mM) were added to the final membrane preparation

[3] Abbreviations: NEM, N-ethylmaleimide; [^{3}H]-HBI, (±)[^{3}H]hydroxybenzylisoproterenol; [^{3}H]DHA, (−)[^{3}H]dihydroalprenolol; Gpp(NH)p, guanyl-5′-ylimidodiphosphate; EDTA, (Ethylenedinitrilo)-tetraacetic acid; DCCD, N,N′ dicyclohexylcarbodiimide; POMB, p-hydroxymercuribenzoate.

immediately prior to incubation in the binding assays (22). The binding incubation was terminated by the addition of 5 ml of cold Buffer A, pouring the diluted sample over a Whatman GF/C glass fiber filter and washing the filter, under vacuum, with an additional 10–15 ml of Buffer A. Unless otherwise stated, binding described in the text and figures refers to specific binding defined as the amount of total binding of the radioligand that is competed for by 10 μM (±)propranolol. Specific binding for [^{3}H]HBI and [^{3}H]DHA was 65–75% and 90–95% of the total binding respectively.

Adenylate cyclase assays. Adenylate cyclase assays were performed as described previously (18) using 50 μl incubations containing 0.12 mM ATP and 0.06 mM GTP. Protein was determined by the method of Lowry (28).

Desensitization of frog erythrocytes. The intact frog erythrocytes were incubated for 4 hours at 23° with or without 0.1 mM (–)isoproterenol (±)bitartrate as described previously (13, 14). A crude membrane fraction was prepared as described previously (17) with the modification that "cyclase buffer" was replaced by Buffer A. The radioligand binding assays and adenylate cyclase assays were performed as described above using 0.4–0.6 mg/ml of crude membrane protein.

RESULTS

Effects of N-ethylmaleimide on high affinity agonist binding. N-ethylmaleimide (NEM) is a reagent that reacts specifically and irreversibly with free sulfhydryl residues of proteins (29). It has been shown that both bacterial (30) and mammalian (26, 31–34) adenylate cyclases possess an essential sulfhydryl group, and in the case of the glucagon-sensitive adenylate cyclase complex in rat liver membranes, the reactivity of the enzyme sulfhydryl was modulated by the occupancy of the receptor by glucagon (33, 34). Previous studies have also shown that chemical modification of disulfide bridges (35) or sulfhydryl groups (36) in *beta*-adrenergic systems alters the binding characteristics of the receptor. We have employed NEM to investigate the relationship between agonist-receptor binding and adenylate cyclase activity in frog erythrocyte membranes (Fig. 1) with the following results:

1) NEM completely inactivates adenylate cyclase in frog erythrocyte membranes at concentrations below 1 mM.

2) NEM also decreases the affinity of the agonist [^{3}H]HBI for the *beta*-adrenergic receptor in a dose dependent way but only at NEM concentrations >1 mM.

3) The binding of the antagonist ligand (–)[^{3}H]dihydroalprenolol ([^{3}H]DHA) to the receptor was unperturbed by NEM concentrations up to 10 mM.

Qualitatively similar results were obtained using the more potent sulfhydryl reagent p-hydroxymercuribenzoate (POMB). Antagonist [^{3}H]DHA binding was unaffected but the affinity of the receptor for the agonist [^{3}H]HBI decreased after expo-

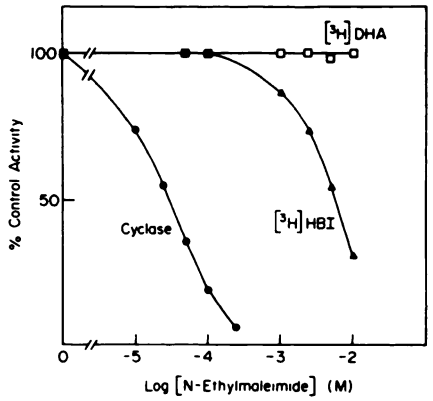

FIG. 1. *Effect of NEM on adenylate cyclase activity and on the binding of [^{3}H]DHA and [^{3}H]HBI*

Frog erythrocyte membranes were incubated with the indicated concentrations of NEM for 10 min at 25°. At the end of the incubation period the reaction was stopped by the addition of an equal concentration of dithiothreitol and the membranes washed twice in Buffer A. The membranes were resuspended in this buffer and assayed for adenylate cyclase activity (●) and the binding of [^{3}H]DHA (□) and [^{3}H]HBI (▲) as described in METHODS. The concentrations of [^{3}H]DHA and [^{3}H]HBI in the binding assays were 3 nM and 7 nM respectively. Control activities refer to the NaF stimulated adenylate cyclase activity (8482 pmol cAMP/min/mg of protein), [^{3}H]DHA binding (655 fmol/mg of protein) and [^{3}H]HBI binding (299 fmol/mg of protein) in membranes that were not exposed to NEM. Each point represents the mean of duplicate (●) or triplicate (□, ▲) determinations in two experiments.

sure of the membranes to POMB > 10 μM, a concentration of this reagent that already inhibits adenylate cyclase activity more than 50% (data not shown). The data indicate that individual sulfhydryl groups modulate enzyme catalytic activity and agonist affinity for the receptor. The sulfhydryl modulating receptor affinity discriminates between agonist and antagonist agents. Exposure of erythrocyte membranes to NEM decreased the affinity of the receptor for the agonist ligands [^{3}H]HBI (Fig. 1) and (-)isoproterenol (Fig. 2) but did not alter antagonist [^{3}H]DHA binding (Fig. 1). Additionally, NEM did not affect the ability of the antagonist propranolol to compete for [^{3}H]DHA binding (data not shown).

To examine the effects of NEM on the ability of guanine nucleotide to alter agonist binding, competition binding experiments were performed utilizing [^{3}H]DHA as the radioligand and (-)isoproterenol as the competing agonist (Fig. 2). Membranes reacted with increasing concentrations of NEM displayed a progressively lower affinity for the agonist, but in all cases (-)isoproterenol exhibited the same low affinity for the receptor in the presence of the nonhydrolyzable guanine nucleotide analog Gpp(NH)p. The concentration dependency of Gpp(NH)p for reducing agonist binding affinity was not altered by NEM treatment (data not shown).

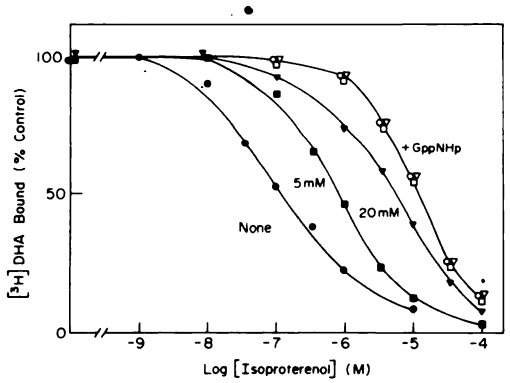

FIG. 2. *(-)Isoproterenol competition for [^{3}H]DHA binding to frog erythrocyte membranes pretreated with NEM*

Membranes were incubated with 0, (●, ○), 5 mM (□, ■), and 20 mM (▼, ▽) NEM for 10 min at 25°. At the end of the incubation period the reaction was stopped by the addition of an equal concentration of dithiothreitol and the membranes were washed twice. Binding assays were carried out as described in METHODS in the absence (filled symbols) and in the presence (unfilled symbols) of 0.1 mM Gpp(NH)p. Control binding refers to the binding of [^{3}H]DHA (699 fmol/mg of protein) in membranes that were incubated in the absence of NEM. Control binding was the same in the presence of Gpp(NH)p. Each point represents the mean of duplicate determinations from two (■, □, ▼, ▽) or four (●, ○) experiments.

A number of experiments were conducted to determine which component of the receptor-adenylate cyclase complex contains the sulfhydryl group modulating agonist affinity. To do this, membranes were reacted with 5 mM NEM and radiolagand binding examined. This concentration of NEM completely inactivates the cyclase catalytic unit and decreases [^{3}H]HBI binding about 40%, while [^{3}H]DHA antagonist binding is unaffected (Table 1).

Membranes were preincubated with the antagonist propranolol (10 μM) or with the guanine nucleotide GTP (1 mM) prior to exposure to 5 mM NEM to determine if these agents would alter the reactivity of the sulfhydryl group. Neither propranolol nor GTP altered the characteristic effect of NEM on agonist or antagonist affinity (Table 1). The data suggest that the sulfhydryl group is not at the ligand binding site of the receptor nor at the ligand binding site of the nucleotide regulatory component. Alternatively, if the membranes were preincubated with the agonist [^{3}H]HBI (13 nM) and subsequently reacted with 5 mM NEM, high affinity binding of the agonist is resistant to the effect of NEM (Fig. 3). The amount of [^{3}H]HBI bound is the same in membranes treated with NEM as those incubated without NEM even after several washings. The dissociation rate of [^{3}H]HBI is not increased by NEM treatment. Moreover, agonist dissociation is still sensitive to Gpp(NH)p. Thus, the sulfhydryl group involved in regulating the high affinity agonist binding to the *beta*-adrenergic receptor complex can be protected against the action of NEM by occupying the receptor with an agonist. In contrast, preincubation of the membranes with antagonist or guanine nucleotide did not alter the effect of NEM on agonist affinity.

TABLE 1

Inability of propranolol and GTP to protect high affinity agonist binding against the effect of NEM

Frog erythrocyte membranes were first incubated with 10^{-3} M GTP, or 10^{-5} M propranolol for 10 min at 25°. The groups of preincubated membranes were divided and one portion exposed to 5 mM NEM for 10 min at 25°, while the other portion was incubated with buffer and served as the control. At the end of the second 10 min incubation the NEM reaction was stopped by the addition of an equal concentration of dithiothreitol. The membranes were washed three times and then resuspended for determination of [^{3}H]DHA and [^{3}H]HBI binding. The concentration of [^{3}H]DHA and [^{3}H]HBI in the binding assay was 3 nM and 5 nM, respectively. Control [^{3}H]DHA and [^{3}H]HBI binding was 545 fmol/mg of protein and 396 fmol/mg of protein respectively. The binding of [^{3}H]DHA and [^{3}H]HBI was the same in control membranes and membranes incubated with propranolol or GTP in the absence of NEM. Values presented represent the mean of duplicate determinations from two experiments ± S.E.M.

Preincubation	NEM	% Control Binding	
		[^{3}H]DHA	[^{3}H]HBI
—	5×10^{-3} M	99.9 ± 2.1[a]	58.5 ± 0.9
Propranolol (10^{-5} M)	5×10^{-3} M	97.1 ± 1.6	61.5 ± 6.1
GTP (10^{-3} M)	5×10^{-3} M	99.6 ± 10.2	61.6 ± 1.1

[a] S.E.M.

Effect of NEM on receptor regulation. It can be seen from Figure 1 that the sulfhydryl group associated with adenylate cyclase catalytic activity is >100 fold more sensitive to NEM than the sulfhydryl regulating agonist affinity. Due to the disparate sensitivities of these two functions to NEM it is possible to examine, in whole cells, the role of agonist high affinity state in the absence of adenylate cyclase activity in the process of receptor regulation. Incubation conditions were determined for intact frog erythrocytes (6 mM NEM, 15 min, 37°) that inhibited catalytic activity >95% in membranes subsequently derived from these cells, but that did not impair the ability of the membrane receptors to bind agonist with high affinity. Erythrocytes that had been reacted with NEM were then exposed to (-)isoproterenol (0.1 mM) for 4 hours at 23°. Such prolonged incubations of control cells with (-)isoproterenol have been shown to lead to an apparent decrease in the number of *beta*-adrenergic receptors (13, 14, 17). High affinity agonist binding was determined indirectly by (-)isoproterenol competition of [^{3}H]DHA binding in membranes prepared from NEM-treated and control cells at the end of the 4 hour incubation in the presence and absence of (-)isoproterenol (Fig. 4). Pretreatment of the cells with NEM did not affect the ability of 10^{-6} M (-)isoproterenol to compete with [^{3}H]DHA for occupancy of the *beta*-adrenergic receptor. This indicates that the overall affinity of agonist binding and the extent of high affinity receptor formation by (-)isoproterenol were unaltered in membranes derived from NEM treated cells. In addition, the inhibition of [^{3}H]DHA binding by (-)isoproterenol (10^{-6} M) was reversed to the same extent by Gpp(NH)p. This demonstrates that nucleotide regulation of agonist affinity was unaffected by NEM under these reaction conditions for intact frog erythrocytes.

Following prolonged incubation with (-)isoproterenol, membranes from cells exposed to the agonist, but not reacted with NEM, demonstrated an apparent loss in the number of receptors as assessed by both [^{3}H]DHA and [^{3}H]HBI binding and a decrease in the ability of agonist catecholamine to stimulate adenylate cyclase (Fig. 5A). However, the erythrocytes preincubated with NEM were resistant to (-)isoproterenol induced receptor regulation (Fig. 5B). Additionally cells exposed to NEM were incubated with 10^{-4} M (-)isoproterenol and 5 mM dibutyryl cAMP but the addition of the cAMP analogue did not restore the receptor regulation process (data not shown).

DISCUSSION

Considerable research effort has been devoted to elucidating the molecular mechanism whereby the binding of a hormone to

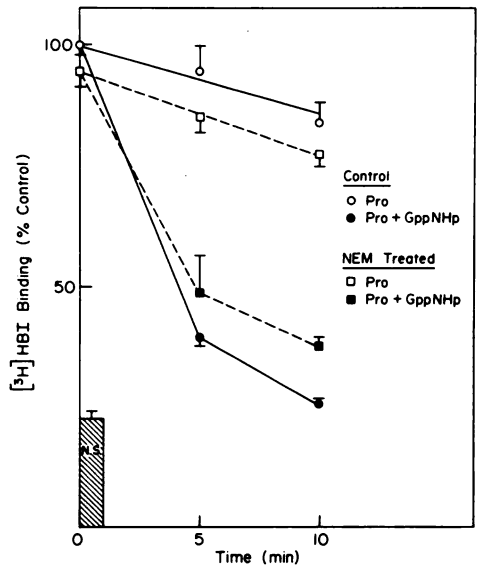

FIG. 3. *Resistance of agonist induced high affinity state to the effect of NEM*

Frog erythrocyte membranes were incubated with [^{3}H]HBI (13 nM) for 20 min at 25°. Specific [^{3}H]HBI binding determined at the end of this incubation was 769 fmoles/mg of protein and represented 78% of the total binding as is indicated on the ordinate. The membranes were washed once to remove free [^{3}H]HBI and the resuspended membranes were divided equally and then incubated in the presence (---) and absence (——) of 5 mM NEM for 10 min at 25°. The NEM was quenched by the addition of an equal concentration of dithiothreitol and the membranes were washed twice. The resuspended membranes were incubated with 10^{-5} M propranolol (unfilled symbols) and 10^{-5} M propranolol plus 10^{-4} M Gpp(NH)p (filled symbols) and total [^{3}H]HBI binding determined at the times indicated. Each point represents the mean and SEM of triplicate determinations from two experiments. N.S. = nonspecific [^{3}H]HBI binding.

its receptor on the cell surface communicates across the plasma membrane to modulate activities within the cell. Recent reconstitution studies on the *beta*-adrenergic coupled adenylate cyclase system utilizing mutants of a S49 murine lymphoma cell line have demonstrated the existence of multiple separate components that are required for receptor-enzyme coupling (26, 27). These components include a hormone binding moiety or receptor, a nucleotide regulatory or coupling component, and a catalytic protein. Because high affinity agonist binding may be related to the ability of agonists to initiate their biological effects, we have used NEM to selectively modify two of the components of the receptor-enzyme complex in frog erythrocyte membranes to determine which functions of this system are necessary for the formation of agonist high affinity state.

It can be seen from Figure 1 that adenylate cyclase activity is about 100 fold more sensitive to NEM than is agonist [^{3}H]HBI binding. Therefore two distinct sulfhydryl groups have been identified with different functions of the receptor-enzyme complex. The NEM dependent decrease in [^{3}H]HBI binding appears to be due to a progressive loss of the ability of agonist to form a high affinity complex with the receptor and the same low affinity state is approached by exposure to NEM as is observed in the presence of guanine nucleotide (Fig. 2). Furthermore, after NEM treatment the curve for (-)isoproterenol competition for [^{3}H]DHA binding is steeper which is also characteristic of guanine nucleotide effects on agonist binding (39). Thus it appears that the reaction of the sulfhydryl group with NEM at concentrations >1 mM affects agonist binding in a fashion very analogous to the addition of guanine nucleotides. These data also demonstrate that catalytic adenylate cyclase activity is not required for the formation of the agonist high affinity binding state and that guanine nucleotide regulation of agonist affinity is likewise independent of enzyme activity. This finding of nucleotide modulation of receptor affinity for agonists in the absence of adenylate cyclase confirms previous observations in the S49 lymphoma cell (36).

The sulfhydryl group modulating agonist affinity appears to be located on the *beta*-adrenergic receptor complex distal to the hormone binding site since the binding of the antagonist [^{3}H]DHA was unaltered by NEM concentrations up to 10 mM (Fig. 1). This is further supported by the observation that occupancy of the receptor by the antagonist propranolol did not alter the subsequent effects of NEM on agonist binding (Table 1). In contrast, the agonist high affinity state, formed by preincubation of membranes with agonist, was completely resistant to NEM and the agonist receptor

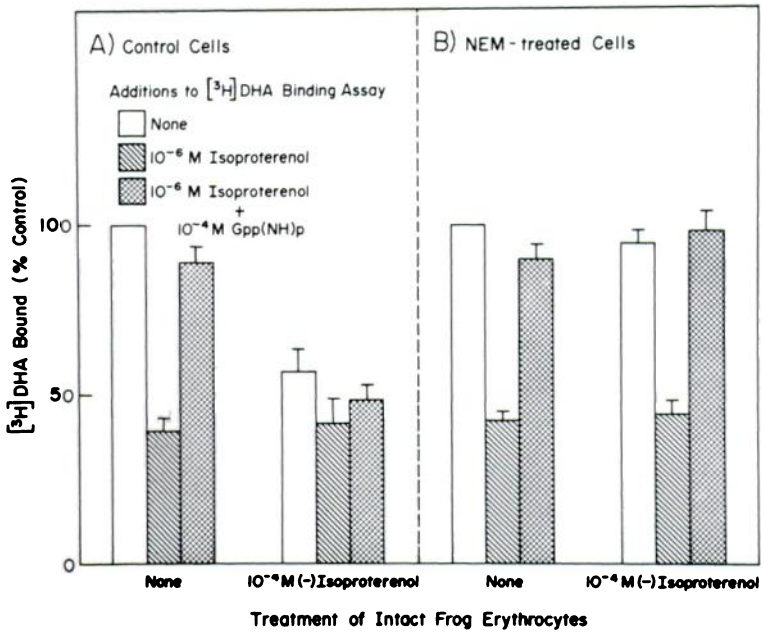

FIG. 4. *Exposure of intact frog erythrocytes to NEM does not affect the ability of (-)isoproterenol to form a nucleotide sensitive high affinity complex with* beta-*adrenergic receptors in erythrocyte membranes*

Frog erythrocytes suspended in buffered amphibian saline (100 mM NaCl, 10 mM Tris-HCl, pH 7.4) were incubated for 15 min at 37° in the absence (Fig. 4A) and presence (Fig. 4B) of 6 mM NEM. The cells were washed 4 times with amphibian saline. The cells were then incubated with 10^{-4} M (-)isoproterenol for 4 hours at 23° as described previously (13, 14). At the end of 4 hours the cells were washed 3 times with amphibian saline to remove the (-)isoproterenol and a crude membrane fraction was prepared (37). These membranes were assayed for [^{3}H]DHA binding in the absence of (-)isoproterenol, in the presence of 10^{-6} M (-)isoproterenol and in the presence of 10^{-6} M (-)isoproterenol plus 10^{-4} M Gpp(NH)p. Control [^{3}H]DHA binding in membranes prepared from cells not incubated with (-)isoproterenol was 100 fmol/mg of protein for both Figs. 4A and 4B. Adenylate cyclase activity in erythrocytes exposed to NEM was inhibited >95% (data not shown). Values shown represent the mean and SEM of triplicate determination from four experiments.

complex remained sensitive to Gpp(NH)p (Fig. 3). These observations can be explained by a model in which the receptor and the nucleotide regulatory site are separate components of the receptor-adenylate cyclase complex. A sulfhydryl group, distinct from the *beta*-adrenergic ligand binding site, modulates agonist binding affinity in a fashion similar to guanine nucleotides and this sulfhydryl is inaccessible to NEM after the formation of the agonist-induced high affinity state. It is this high affinity state that is uniquely sensitive to guanine nucleotides, suggesting that agonist binding may lead to an association of the receptor and nucleotide site components. The agonist induced association of these components appears to protect the agonist-affinity modulatory sulfhydryl group from NEM and permits the action of guanine nucleotides on agonist affinity. The determination of the exact location of the agonist affinity modulating sulfhydryl will require the purification of the components required for high affinity binding. The data is compatible with the sulfhydryl group being located at the receptor-nucleotide component interface, but it could be on either component. Catecholamine stimulated GTPase activity in turkey erythrocyte membranes has been demonstrated to show an increased sensitivity to NEM in the presence of agonists (40). It is unknown if this specific GTPase may also modulate receptor affinity. Moreover, in a recent study the reactive sulfhydryl modulating the inhibition of catecholamine stimulated GTPase appeared to be located on the receptor (41).

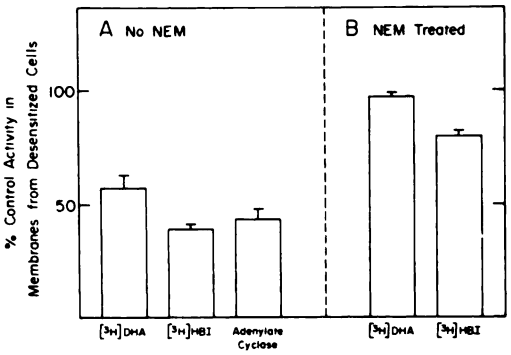

FIG. 5. *Effect of NEM on (-)isoproterenol induced desensitization in frog erythrocytes*

Frog erythrocytes suspended in buffered amphibian saline (100 mM NaCl, 10 mM Tris-HCl, pH 7.4) were incubated for 15 min at 37° in the absence (Fig. 5A) and presence (Fig. 5B) of 6 mM NEM. The cells were washed 4 times with amphibian saline. The cells were then incubated with 10^{-4} M (-)isoproterenol for 4 hours at 23°, as described previously (13, 14). At the end of 4 hours the cells were washed three times in amphibian saline to remove the (-)isoproterenol and a crude membrane fraction was prepared (37). These membranes were then assayed for adenylate cyclase activity, [^{3}H]DHA and [^{3}H]HBI binding as described in METHODS. Control [^{3}H]DHA and [^{3}H]HBI binding in membranes prepared from cells not exposed to (-)isoproterenol was 100 fmol/mg of protein and 98 fmol/mg of protein respectively for Fig. 4A and 100 fmol/mg of protein and 83 fmol/mg of protein, respectively, for Fig. 4B. Adenylate cyclase refers to the enzyme stimulated by 0.1 mM (-)isoproterenol above basal activity and was 403 pmol cAMP/min/mg of protein in membranes prepared from cells incubated in the absence of NEM and (-)isoproterenol. Adenylate cyclase activity in erythrocytes exposed to NEM was inhibited >95% (data not shown). Values shown represent the mean and SEM of triplicate ([^{3}H]DHA and [^{3}H]HBI binding) or duplicate (adenylate cyclase) determinations from four separate experiments.

We favor the receptor moiety as the locus for the sulfhydryl since the dose dependency of guanine nucleotide effects on agonist affinity is unaffected by NEM treatment and preincubation of the membranes with GTP did not significantly alter the reactivity of this sulfhydryl towards NEM (Table 1).

By taking advantage of the widely different sensitivities of adenylate cyclase and agonist affinity to NEM, it has also been possible to investigate the importance of agonist high affinity binding in the process of whole cell receptor regulation in the absence of adenylate cyclase activity. Previous studies have implicated an important role for agonist high affinity binding for the subsequent decrease in receptor number (37). The process of receptor regulation appears to result in a selective loss of high affinity agonist binding sites and treatment of frog erythrocytes with reagents such as N,N'-dicyclohexylcarbodiimide (DCCD), which blocks the formation of agonist high affinity state, also prevents down regulation of receptors (37, 38). These observations indicate a requirement for the ability of agonist to form a high affinity complex with the receptor for these agents to initiate receptor regulation. Shear *et al.* (15) suggested an additional requirement for functional adenylate cyclase to obtain agonist induced receptor refractoriness in the S49 lymphoma cell. Their data were derived from a mutant S49 clone that lacks measurable adenylate cyclase activity but does contain *beta*-adrenergic receptors. When these mutant cells were incubated with (-)isoproterenol no loss in receptor number was observed in radioligand binding studies using [^{125}I]hydroxybenzylpindolol, while the number of receptors in wild type S49 cells did decrease under the same incubation conditions with agonist. Recent experiments, however, have demonstrated that the S49 mutant cell clone employed in the receptor regulation studies is, in fact, devoid of the nucleotide regulatory component of the receptor-cyclase complex, but retains a functional catalytic moiety (26, 27). The receptor regulation experiments utilizing the mutant S49 cells thus suggest an obligatory role for the nucleotide regulatory component in the desensitization process. This observation supports the hypothesis that the ability of agonist to form a high affinity state is necessary to trigger the receptor regulation process and is consistent with the molecular model for the high affinity binding stated above. That is, without the nucleotide regulatory component the S49 cell can neither desensitize in the presence of agonist (15) nor form a high affinity-nucleotide sensitive agonist complex (39).

In the present study NEM was utilized

to dissect the receptor-cyclase complex in a unique way. NEM treatment of frog erythrocytes under appropriate conditions inactivates the cyclase enzyme, but does not interfere with the ability of agonists to form a high affinity complex with the receptor that is fully modulated by guanine nucleotides. This NEM treatment of erythrocytes also prevents agonist-initiated receptor regulation (Fig. 5). Although adenylate cyclase is very sensitive to NEM (Fig. 1), it cannot be ruled out that some other unknown component necessary for the receptor regulation process is also inhibited by the NEM pretreatment. However, it can be concluded that agonist occupancy of a nucleotide-sensitive receptor complex in the absence of a functional adenylate cyclase is insufficient, by itself, to trigger receptor regulation in the whole cell. A possible unifying explanation for the inability of agonists to desensitize the receptors in the mutant S49 cell clone (15) and frog erythrocytes treated with DCCD (37) or NEM is that agonist stimulation of the adenylate cyclase is indeed necessary. A common characteristic of the three studies is that receptor regulation is prevented when agonist-receptor occupancy is not communicated or coupled to cyclase activation independent of whether agonist can form a high affinity receptor complex that is modulated by guanine nucleotides. The desensitization process, therefore, appears to require more than formation of the high affinity state of the receptor. It also appears to depend on productive coupling between agonist occupancy of the receptor and an NEM sensitive component possibly adenylate cyclase.

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