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Inactivation of β-Adrenergic Receptors by *N*-Ethylmaleimide in S49 Lymphoma Cells

Agonist Induction of Functional Receptor Heterogeneity

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SUMMARY

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β-Adrenergic agonists appear to induce a conformational change in 65% of the β-adrenergic receptors in wild-type S49 lymphoma cell membranes as shown by the increased sensitivity of a subpopulation of receptor to inactivation by the alkylating agent *N*-ethylmaleimide (NEM). This inactivation is conveniently monitored through the induction with NEM of a biphasic (−)-isoproterenol displacement curve of [^{125}I]IHYP binding to the receptors. In marked contrast, NEM alone or NEM plus a β-adrenergic antagonist has no effect on receptor binding. The final percentage (65%) of total receptor sites inactivated with agonist plus NEM is not dependent on the concentration of NEM used or on the time of exposure. Agonist plus NEM also causes inactivation of about 75% of the receptors in the S49 lymphoma β_d clone, which has about 25% of the β-adrenergic receptor density of wild-type cells. However, agonist plus NEM treatment does not affect receptor binding in *unc* and *cyc*$^-$ S49 cell membranes, in which the receptors are functionally uncoupled from the adenylate cyclase complex. Further, agonists plus NEM also cause inactivation of 45 to 60% of the β-adrenergic receptors in turkey erythrocyte membranes, but have no effect on solubilized receptors from the same cell. These findings with variant S49 lines and turkey erythrocytes suggest that the ability of agonists to produce a conformational change of β-adrenergic receptors may be dependent on the ability of these receptors to interact with the adenylate cyclase complex and specifically to interact with the guanine nucleotide regulatory protein. The fact that only a given fraction of the total receptor population can undergo a conformational change in the investigated membranes and that the properties of the agonist/NEM-sensitive population of receptors but not the insensitive population are similar to those of receptors which activate adenylate cyclase suggests that, for structural or stoichiometric reasons, only part of the receptor population can be coupled to the cyclase complex in the presence of agonists. Thus agonist appears to induce a quantifiable, functional heterogeneity in an apparently molecularly homogeneous receptor population.

INTRODUCTION

Specific, high-affinity binding of radiolabeled β-adrenergic ligands has proven to be a useful tool for the direct identification and characterization of β-adrenergic receptors in many tissues. In this context, several recent studies have shed light on marked structural and functional differences between interactions of agonists and antagonists with the receptor. These findings include: (a) differences in molecular size between the agonist- and antagonist-labeled forms of the frog erythrocyte β-adrenergic receptor (1); (b) the specific ability of agonists to cause an apparent decrease in receptor density in intact cell preparations (desensitization) (2–6); (c) an apparent increase specifically in agonist versus antagonist affinity for binding to β-adrenergic receptors caused by magnesium ions (7, 8) and the reversal of this effect by guanine nucleotides (7); (d) differences in the thermodynamic

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parameters of agonist versus antagonist interactions with the β-adrenergic receptor in turkey erythrocyte membranes (9); and (e) the specific ability of agonists to cause a conformational change of the turkey erythrocyte β-adrenergic receptors (10, 11), as measured by an increased sensitivity toward alkylation by *N*-ethylmaleimide (NEM).[1]

Interestingly, several of these agonist-specific actions affect only part of the β-adrenergic receptor population. These include desensitization phenomena (2, 4, 5), induction of high affinity for agonists in the presence of Mg^{2+} (2), and inactivation of agonist-bound receptors by NEM (10, 11). These data are indicative of an apparent heterogeneity among the β-adrenergic receptors in isolated cell systems such as turkey and frog erythrocytes as well as cultured S49 lymphoma cells. In contrast to the recent demonstration of the coexistence of β_1- and β_2-adrenergic receptors in tissues such as heart, lung, and brain (12–15), no evidence has yet been found for the presence of more than one receptor subclass (i.e., β_1 versus β_2) in these isolated homogeneous cell systems. This additional receptor heterogeneity toward agonists is presumably therefore either the result of differences in receptor environment or due to inherent properties of the β-adrenergic receptor. It might be envisaged that differences in lipid environment or nonstoichiometric coupling between the receptor and other components of the adenylate cyclase system could differentially affect agonist–receptor interaction. Alternative explanations might also include the existence of receptor multimers, of multiple binding sites per receptor molecule, or of more than a single molecular species of receptor.

To gain more information concerning the basic mechanisms underlying this receptor heterogeneity, we have investigated the reactivity of the alkylating agent NEM toward agonist-occupied receptors in S49 lymphoma cell membranes. The considerable advantage of S49 lymphoma cells for the purpose of this study resides in the availability of several variant clones possessing specific defects in the receptor cyclase complex (16) including two variants which contain β-adrenergic receptors not functionally coupled to the adenylate cyclase. In the present report, we show that NEM inactivates approximately 65% of the agonist-bound receptors in wild-type and β_d S49 cell membranes, but not in *unc* and *cyc*$^-$ variants. Agonists plus NEM also no longer inactivate turkey erythrocyte β-adrenergic receptors after solubilization. These data suggest that the β-adrenergic receptor heterogeneity in the membranes is related to the ability of a specific subpopulation of these receptors to couple to the adenylate cyclase complex, specifically to the guanine nucleotide coupling protein.

MATERIALS AND METHODS

Materials. The following were obtained as generous gifts: (±)-hydroxybenzylpindolol (Sandoz), (−)-propranolol (Ayerst), (+)-epinephrine bitartrate (Sterling Winthrop), (±)-alprenolol hydrochloride (Ciba-Geigy), Ro 20-1724 (Hoffman-La Roche). [^{125}I]Na was purchased from Amersham/Searle and (−)-[^{3}H]dihydroalprenol (33 Ci/mmol) from New England Nuclear Corp. Other compounds were obtained from either Sigma, Boehringer Mannheim, J. T. Baker, or Grand Island Biochemical.

Membrane preparations. All S49 lymphoma cell clones were routinely grown in stationary suspension or in spinner culture at 37°C in Dulbecco's modified Eagle's medium (4.5 g/liter of D-glucose) containing 8 to 10% heat-inactivated horse serum. Preparation of plasma membranes from wild-type and variant S49 cells was performed as previously described (17) except that membranes were suspended for storage in 20 mM sodium–Hepes (pH 8 at 30°C) plus 0.1 mM EDTA. Membranes were stored at −90°C until use. Turkey erythrocyte membranes were prepared according to Øye and Sutherland (18), with small modifications as described elsewhere (19). Membranes were suspended in 10 mM Tris–HCl (pH 7.4), 145 mM NaCl, 2 mM $MgCl_2$, 10% glycerol (v/v) and stored in liquid nitrogen until use. Solubilization of the erythrocyte membranes (3 mg/ml) with digitonin (Merck A. G.; 0.25%, w/v) was performed in 75 mM Tris–HCl (pH 7.4) plus 25 mM $MgCl_2$, as described (20). Solubilization of the β-adrenergic receptors occurred with a yield of 32%. Protein was assayed by the method of Lowry *et al.* (21) using bovine serum albumin as standard.

Membrane pretreatment with β-adrenergic agents and NEM, and binding of [^{125}I]IHYP. In previous studies (10, 11), we have demonstrated that, only in the presence of β-adrenergic agonists, the alkylating agent NEM inactivates 45 to 60% of the β-adrenergic receptors in turkey erythrocyte membranes. This phenomenon was measured by preincubating membranes with agonist plus NEM, removal of these compounds by three washes (centrifugation and resuspension in fresh buffer), and measurement of (−)-[^{3}H]dihydroalprenolol binding to the remaining receptor sites. Due to the high centrifugational forces used during preparation of S49 membranes (17) and the slow dissociation rate constant for [^{125}I]IHYP, the above experimental approach was modified in order to avoid the wash step. The new approach determined [^{125}I]IHYP-specific binding after preincubation of the membrane with increasing concentrations of β-adrenergic agonists in the absence and presence of NEM. When the experiments are performed in the absence of NEM, the data presented in Figs. 1, 3, and 4 are agonist displacement curves for [^{125}I]IHYP binding. In the presence of NEM, however, the data no longer represent true agonist displacement curves. They reflect the ability of a given concentration of agonist to promote receptor inactivation by NEM (at low agonist concentrations)[2] and agonist displacement of [^{125}I]IHYP binding

[1] Abbreviations used: NEM, *N*-ethylmaleimide; [^{125}I]IHYP, [^{125}I]iodohydroxybenzylpindolol; n_H, Hill coefficient.

[2] We show in Results that the effect of agonists plus NEM is to inactivate (i.e., decrease the density of) β-adrenergic receptors without affecting the affinity of the remaining receptor sites for [^{125}I]IHYP. As discussed in Ref. 11 and shown subsequently, the inactivation process is irreversible and affects only 65% of the receptor sites in S49. The ultimate extent of inactivation is independent of the time of preincubation or the concentrations of agonist and NEM; however, the rate of inactivation is time and concentration dependent. Accordingly, due to the fast reversibility and continuous equilibrium of the agonist–receptor interaction, agonist concentrations that cause occupation of only a

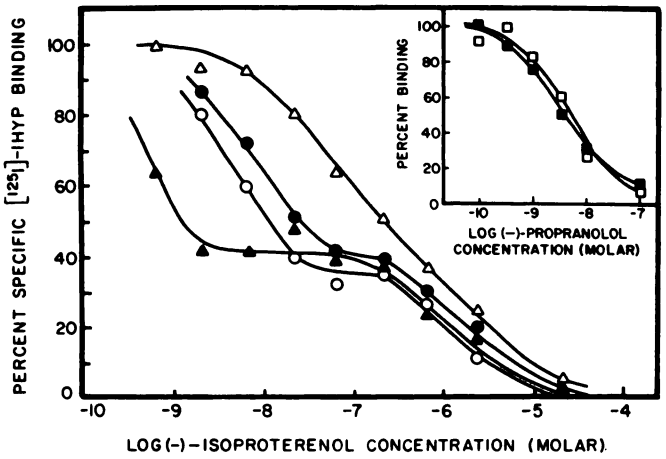

FIG. 1. *Effect of agonist and antagonist on [^{125}I]IHYP binding to β-adrenergic receptors at constant NEM concentration*

Wild-type cell membranes were preincubated with 1.67 mM NEM and increasing concentrations of (−)-isoproterenol for 0 (●), 10 (○), or 30 min (▲) at 30°C, after which each sample was diluted 1.67-fold and incubated with 100 pM [^{125}I]IHYP for an additional 30 min. Specific binding of [^{125}I]IHYP was defined as described in Materials and Methods. Control (100%) binding was always measured in the presence of buffer only. Nonspecific and specific [^{125}I]IHYP binding are not affected by NEM for any length of preincubation (not shown). When performed in the absence of NEM, the same experiments yielded superimposable (−)-isoproterenol displacement binding curves for different preincubation times (△, data shown only for 10-min preincubation with the agonist). Inset: Membranes were preincubated for 10 min with increasing concentrations of (−)-propranolol in the absence (□) or presence (■) of NEM. [^{125}I]IHYP binding to each sample was performed as described above.

for the agonist plus NEM-resistant receptor population (at high concentrations). The steep parts of these curves are separated by a horizontal plateau, which reflects full inactivation of the sensitive receptor population by NEM and no pronounced competition between the agonist and [^{125}I]IHYP for binding to the resistant receptors. Since the agonist plus NEM effect is solely on receptor density,[2] the fraction of sensitive receptors can be quantitated as $(B_{max} - B_p)/B_{max}$, where B_{max} is binding of [^{125}I]IHYP in the absence of agonist and B_p is plateau binding in the presence of agonist plus NEM. Unless otherwise stated, S49 lymphoma cell membranes (0.5 to 1.2 mg membrane protein/ml) were preincubated with increasing concentrations of β-adrenergic agent in the absence or presence of NEM at 1.67 times the final concentration indicated for 10 min at 30°C in a total volume of 60 μl. After 10 min, 40 μl of [^{125}I]IHYP solution was added to the medium. Incubation with the tracer in a final volume of 100 μl was then continued for 30 min at 30°C. The final composition of the medium was 50 mM sodium–Hepes (pH 8 at 30°C), 10 mM $MgSO_4$, 0.1 mM Ro 20-1724, 3 mM potassium phosphoenolpyruvate, 10 μg/ml of pyruvate kinase, 0.1 mM sodium ascorbate, 0.1 mg/ml of bovine serum albumin, 0.5 mM ATP (GTP free), 100 pM [^{125}I] IHYP, and 0.3 to 0.7 mg/ml membrane protein. These conditions are identical to those used for measuring adenylate cyclase activity. Identical results are obtained when assays are performed in the presence of Hepes and Mg^{2+} only. [^{125}I]IHYP was synthesized and its binding was measured as described (22). Specific binding is defined as the difference between the amount of [^{125}I]IHYP bound in the absence and presence of 1 μM (−)-propranolol, and amounted to 85–95% of the total binding in wild-type, *cyc*$^-$, and *unc* membranes and 65% in β_d membranes. Nonspecific binding was unaffected under any condition of incubation with or without agonist and/or NEM. The K_D for binding was calculated from the concentration of drug inhibiting 50% of the [^{125}I]IHYP binding as previously described (7).

(−)-[^{3}H]DHA binding to β-adrenergic receptors in particulate and solubilized turkey erythrocyte membranes. Due to the high reactivity of NEM toward the turkey erythrocyte β-adrenergic receptors in the presence of agonists (10, 11) and the rapid reversibility of (−)-[^{3}H]DHA binding at 30°C (19), the preincubation phase used with S49 membranes could be omitted for these experiments. Accordingly, turkey erythrocyte membranes (2 mg/ml) were incubated for 12 min at 30°C with 10 nM (−)-[^{3}H]DHA, 1 mM NEM, and the indicated concentrations of (−)-isoproterenol in 75 mM Tris–HCl (pH 7.4) plus 25 mM $MgCl_2$ in a final volume of 200 μl. Incubation of solubilized membranes was performed under the same conditions in a final volume of 400 μl. (−)-[^{3}H]DHA binding was measured by a combination of polyethylene glycol precipitation and filtration (for soluble membranes) or filtration only (for particulate membranes), as described (20). Specific binding was defined as the difference between the amount of (−)-[^{3}H]DHA bound in the absence and the presence of 2.5 μM (±)-alprenolol and was 85 and 70% of the total binding to particulate and soluble membranes, respectively.

β-Adrenergic agonists and/or NEM did not affect nonspecific binding of either [^{125}I]IHYP or (−)-[^{3}H]DHA at the concentrations used in this study. In general, all experiments were performed at least twice with replicate samples within experiments agreeing within ±5%. Due to small variations of absolute binding density between membrane preparations, only single, representative experiments are shown unless noted.

RESULTS

Inactivation of Agonist-Bound β-Adrenergic Receptors by NEM in Wild-Type S49 Lymphoma Cell Membranes

In previous studies (7, 17) we have shown that increasing concentrations of β-adrenergic agonists (e.g., (−)-isoproterenol) cause a shallow displacement curve of [^{125}I]IHYP binding to wild-type S49 lymphoma cell membranes when the incubations are conducted in the presence of free Mg^{2+} ions. The (−)-isoproterenol displacement curves are superimposable whether or not the incubation phase with [^{125}I]IHYP is preceded by incubation of the membranes with (−)-isoproterenol (for 10 min as shown in Fig. 1 or up to 30 min, not shown). However, when the same experiments are performed in the presence of NEM, the displacement curves are clearly

small fraction of the receptors can promote maximal (65%) receptor inactivation provided the NEM concentration is sufficiently high and the preincubation time is sufficiently long.

biphasic (Fig. 1). This phenomenon is time dependent, since preincubation with agonist plus NEM for increasing periods of time (0, 10, and 30 min) causes a gradual leftward shift of the initial portion of the displacement curves (Fig. 1). This phenomenon is also agonist specific, since NEM affects neither [^{125}I]IHYP binding alone (see below) nor displacement of bound [^{125}I]IHYP by increasing concentrations of the antagonist (−)-propranolol (Fig. 1, inset).

The leftward shift of the initial portion of the (−)-isoproterenol displacement curves in the presence of NEM could be explained by three potential mechanisms: (a) inactivation of about 65% of the agonist-bound receptors by NEM, (b) an NEM-induced increase in agonist affinity for 65% of the sites, or (c) an agonist plus NEM-induced decrease in the affinity of [^{125}I]IHYP for total receptor or a specific subpopulation. The latter two explanations can, however, be discarded on the basis of the following observations. First, Scatchard analysis (24) of [^{125}I]IHYP saturation binding shows that membrane pretreatment with 20 nM (−)-isoproterenol plus NEM causes a net decline in the amount of receptor sites, without significant effect on the affinity of the tracer for the 35% of sites remaining (Table 1). These same data also demonstrate that NEM alone has no effect on [^{125}I]IHYP binding. Second, the kinetic data presented in Fig. 1 are compatible with the hypothesis of an NEM-induced increase in agonist affinity only if one assumes that this effect is time dependent. This explanation is highly unlikely since preincubation of the membranes with NEM alone for either 0 or 30 min, followed by incubation with [^{125}I]IHYP in the presence of increasing concentrations of agonist, yields superimposable displacement binding curves (data not shown). Therefore, direct inactivation by NEM of a subpopulation of 65% of the agonist-bound

TABLE 1

Characteristics of [^{125}I]IHYP binding to wild-type S49 lymphoma cell membranes after pretreatment with agonist and/or NEM

Cell membranes were preincubated with buffer alone, or with 33 nM isoproterenol and 1.67 mM NEM either alone or in combination, for 15 min at 30°C, after which each sample was diluted 1.67-fold and incubated with 10 concentrations of [^{125}I]IHYP (10–300 pM) for an additional 30 min. The preincubation conditions were sufficient to fully inactivate agonist/NEM-sensitive receptors. Specific binding of [^{125}I]IHYP was defined as described in Materials and Methods. The total amount of receptor sites (B_{max}) and the equilibrium dissociation constant for binding (K_D) were calculated by Scatchard analysis (24) of the saturation binding data. A single experiment is shown since different membrane preparations show small variations in absolute affinity of IHYP and receptor density (17). The uncertainty in the binding parameters within a single experiment (by analysis of confidence limits) is routinely ±10%.

Membrane pretreatment with	[^{125}I]IHYP binding characteristics	
	B_{max}	K_D
	fmol/mg protein	*pM*
Buffer alone	205	85
NEM	190	95
(−)-Isoproterenol	180	205
(−)-Isoproterenol plus NEM	65	110

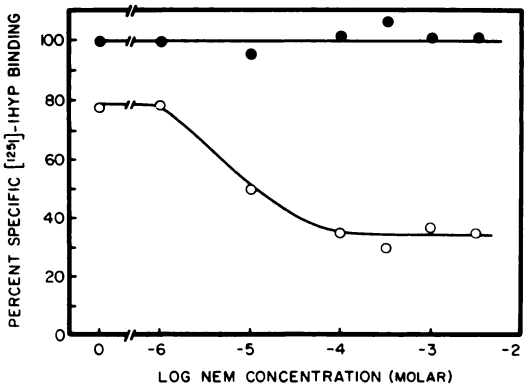

FIG. 2. *Effect of NEM on [^{125}I]IHYP binding to β-adrenergic receptors at constant agonist concentration*

Wild-type cell membranes were preincubated with increasing concentrations of NEM in the absence (●) or presence (○) of 33 nM (−)-isoproterenol for 10 min at 30°C. [^{125}I]IHYP binding to each diluted sample was then performed as described in the legend to Fig. 1. Final NEM concentrations are shown on the abscissa. Specific and nonspecific [^{125}I]IHYP binding are not affected at any NEM concentration used.

receptors is the most plausible interpretation of the data presented in Fig. 1 and Table 1. Further confirmation of this interpretation derives from the observation that no time-related difference in the absolute amount (65%) of inactivated receptor sites or in the relative amount (about 2:1) of the agonist/NEM-sensitive and -insensitive receptor populations can be observed (Fig. 1). The absolute proportion of agonist/NEM-sensitive receptor sites is also independent of the concentration of NEM used (Fig. 2). While concentrations of NEM alone up to 3 mM do not directly affect [^{125}I]IHYP binding, Fig. 2 shows that in the presence of 20 nM (−)-isoproterenol, this alkylator is able to cause a dose-dependent decrease in binding which, under these conditions, is maximal at 100 μM NEM and plateaus at about 65% of the control. The [^{125}I]IHYP binding versus agonist concentration curves obtained after membrane preincubation with agonist plus NEM (Fig. 1) thus reflect the ability of agonists to promote receptor inactivation by NEM at low agonist concentrations and true displacement of [^{125}I]IHYP binding to the remaining, agonist plus NEM-resistant receptor population at higher agonist concentrations.

In a previous study (11), it was demonstrated that β-adrenergic agonists must be physically associated with the turkey erythrocyte β-adrenergic receptor in order to promote inactivation by NEM. To test the validity of this mechanism for the S49 lymphoma system, membranes were preincubated with increasing concentrations of various agonists, in the absence or presence of NEM. Agonists alone inhibit [^{125}I]IHYP binding with the decreasing order of potencies (−)-isoproterenol > (−)-epinephrine > (−)-norepinephrine > (+)-epinephrine (Fig. 3, inset), which is typical for a β_2-adrenergic receptor system (25). In the presence of NEM all agonist displacement curves become biphasic (Fig. 3). However, both portions of the biphasic displacement curves display the same potency ratio (as well as stereoselectivity) as observed in the absence of NEM. This correlation supports the contention that NEM inactivates the S49 lymphoma

β-adrenergic receptors only when receptor is bound by agonist. The data further suggest that on pharmacological grounds, agonist/NEM-sensitive and -resistant receptor subpopulations have the same specificity, i.e., both subpopulations are β_2-adrenergic receptors (Fig. 3).

Effect of Agonists Plus NEM on β-Adrenergic Receptors of Variant S49 Lymphoma Cell Lines

Variant S49 cell lines (*unc* and *cyc*$^-$) have been cloned which contain a normal density of β-adrenergic receptors, but display no catecholamine-stimulated adenylate cyclase activity (16). Since in these cell lines, the β-adrenergic receptors are at least functionally uncoupled from the catalytic subunit–guanine nucleotide regulatory protein(s) complex, they constitute a suitable material for the investigation of any relationship between coupling and the ability of NEM to inactivate agonist-bound receptors. Binding data with *unc* and *cyc*$^-$ membranes indicate that, in contrast with the wild-type receptors (Fig. 4A), NEM does not affect agonist displacement of [^{125}I]IHYP binding (Figs. 4C and D). Therefore we conclude that the agonist-bound receptors must be coupled to one or more additional components of the cyclase complex in order to be inactivated by NEM. Johnson *et al.* (26) have recently reported the selection of S49 lymphoma cell clones which contain a reduced density of β-adrenergic receptors, designated β_d. Figure 4B shows that about 75% of the remaining β-adrenergic receptor binding sites in the β_d clone are inactivated by agonist/NEM. This is not significantly different from the behavior of wild-type receptors. (The relatively low density of receptors in β_d and consequent higher nonspecific binding in β_d membranes make quantitative comparisons of wild-type and β_d binding difficult.) The major point of the experiment is that the β_d clone's receptors exhibit the same heterogeneity as wild-type receptors.

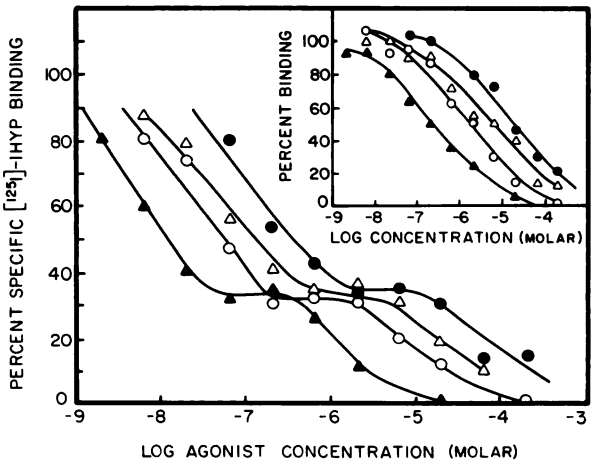

FIG. 3. *The effect of various agonists on [^{125}I]IHYP binding to β-adrenergic receptors at constant NEM concentration*

Wild-type cell membranes were preincubated with increasing concentrations of (−)-isoproterenol (▲), (−)-epinephrine (○), (−)-norepinephrine (△), and (+)-epinephrine (●) in the presence of 1.67 mM NEM for 10 min at 30°C. [^{125}I]IHYP binding to each diluted sample was then performed as described in the legend to Fig. 1. Inset: The same experiments, performed in the absence of NEM.

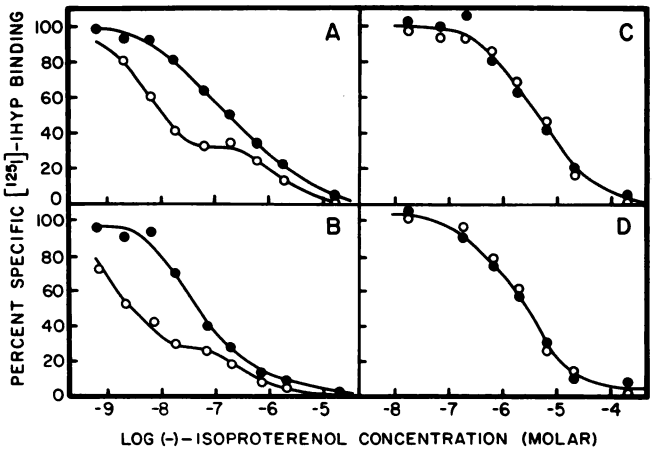

FIG. 4. *Inactivation of agonist-bound receptors by NEM in variant S49 cell types*

Wild-type (A), β_d (B), *unc* (C), and *cyc*$^-$ (D) membranes were preincubated with increasing concentrations of (−)-isoproterenol in the absence (●) or presence (○) of 1.67 mM NEM for 10 min at 30°C. Subsequent [^{125}I]IHYP binding to each sample was performed as described in the legend to Fig. 1. The β-adrenergic receptor density in the β_d cell membranes (58 fmol/mg protein) was 28% of the receptor density in the wild-type membranes (205 fmol/mg protein), as obtained by Scatchard analysis of [^{125}I]IHYP saturation binding (not shown).

Effect of Agonist/NEM on Membrane-Bound and Solubilized Turkey Erythrocyte β-Adrenergic Receptors

Digitonin-solubilized turkey erythrocyte β-adrenergic receptors retain their major pharmacological binding characteristics (23). These solubilized receptors are no longer coupled to other components of the cyclase system since they no longer stimulate adenylate cyclase activity (20) and can be completely freed from cyclase activity and guanine nucleotide binding sites by affinity chromatography (20, 23). Based on the information obtained by investigating the variant S49 cell lines, one would therefore expect that the ability of NEM to inactivate agonist-bound receptors should be lost after solubilization. This is demonstrated in Fig. 5. Whereas NEM inactivates about 50% of the agonist-bound receptor sites in turkey erythrocyte membranes (10, 11), no NEM effect is observed upon the (−)-isoproterenol displacement curve of (−)-[^{3}H]DHA binding to the solubilized receptors (Fig. 5B) in contrast to membrane-bound receptors (Fig. 5A).

Affinity of β-Adrenergic Agonists for Agonist/NEM-Sensitive and -Resistant Receptors

(−)-Isoproterenol displacement binding data obtained in the absence of NEM shown in Fig. 1 reveal an apparent Hill coefficient (n_H) of 0.62 ($r = 0.984$) (Fig. 6). Assuming that this apparent negative cooperativity might be related to the ability of only part of the receptors to be coupled to the cyclase, we have investigated the (−)-isoproterenol binding characteristics for the agonist/NEM sensitive and -resistant receptor populations. In contrast with (−)-isoproterenol binding to the total receptor population, interaction with the resistant sites occurs with a $n_H = 1.1$ ($r = 0.992$) and with a much higher apparent K_D (2 μM) (Fig. 6). These characteristics are

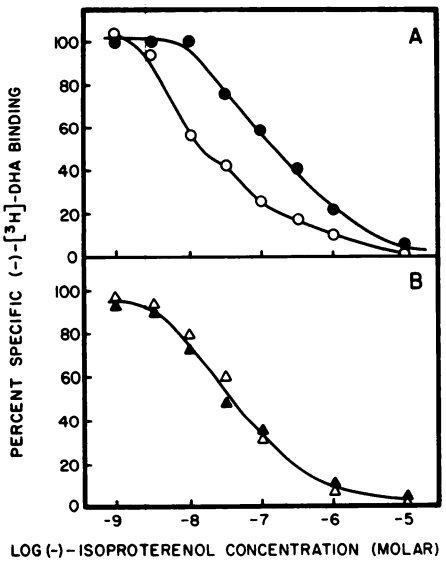

FIG. 5. *Inactivation of agonist-bound receptors by NEM: effect of solubilization*

Particulate (A) and digitonin-solubilized (B) turkey erythrocyte membranes were incubated with 10 nM (−)-[^{3}H]dihydroalprenolol and the indicated concentrations of (−)-isoproterenol in the absence (●, ▲) or presence (○, △) of 1 mM NEM for 12 min at 30°C. Specific (−)-[^{3}H]dihydroalprenolol binding was measured as described in Materials and Methods and was not affected by NEM in either particulate or solubilized membranes. Solubilization of the receptors occurred with a yield of 32%.

similar to those obtained for (−)-isoproterenol displacement binding to the variant cell lines, i.e., $n_H = 0.80$, $K_D = 2$ μM ($r = 0.997$) for *unc* and $n_H = 0.88$, $K_D = 1$ μM ($r = 0.998$) for *cyc*$^-$ (Fig. 6). Thus agonist/NEM-resistant receptors have similar agonist binding parameters, regardless of whether they are a receptor subpopulation in wild-type membranes or form the entire population in the *unc* and *cyc*$^-$ cell lines. An (−)-isoproterenol displacement binding curve for the agonist/NEM-sensitive sites in wild-type membranes can be obtained by subtracting [^{125}I]IHYP binding to the resistant sites from binding to the total receptor population at corresponding (−)-isoproterenol concentrations. The Hill plot of this curve is also presented in Fig. 6 and gives $n_H = 0.95$, $K_D = 0.03$ μM ($r = 0.996$). The apparent K_D and n_H of this subpopulation are similar to the parameters measured for (−)-isoproterenol activation of adenylate cyclase in S49 membranes (7, 17). This analysis (Fig. 6) suggests that (−)-isoproterenol binds with high affinity and without noticeable cooperativity to the agonist/NEM-sensitive receptor population (65% of the total population) and with low affinity and again without significant cooperativity to the resistant population (35% of the total population) in wild-type membranes. (This analysis explains also why low concentrations of agonist actually appear to increase slightly the K_D of [^{25}I]IHYP for binding to the total receptor population, whereas this effect is not present in the agonist/NEM-resistant population (Table 1).)

DISCUSSION

By use of the sensitivity toward NEM alkylation as a structural probe, we have recently demonstrated that β-adrenergic agonists cause a rapid and reversible change of the β_1-adrenergic receptors in turkey erythrocyte membranes which is presumably conformational in nature (10, 11). The observation that this conformational change affected only a subclass of the receptor population (45 to 60%) constituted a striking, but unexplained result (10). In the present report, we show that NEM also inactivates agonist-bound β_2-adrenergic receptors in wild-type S49 lymphoma cell membranes. It is likely, therefore, that agonists cause similar conformational changes in both β_1- and β_2-adrenergic receptors. Further, NEM inactivates only 65% of the agonist-bound receptor sites in wild-type S49 cell membranes. The absolute extent of this inactivation is not affected by the time of exposure (Fig. 1) or caused by a limited supply of agonist (Figs. 1 and 3) or NEM (Fig. 2), in agreement with the data on turkey erythrocyte receptors.

We suggest that this ability of β-adrenergic ligands to cause a conformational change of their receptors is closely related to (a) their ability to activate adenylate cyclase and (b) the ability of the receptor to be functionally coupled to the enzyme. In Fig. 3, it is shown that inactivation by NEM occurs in the presence of full agonists such as (−)-isoproterenol, (+)- and (−)-epinephrine, and (−)-norepinephrine, while this process is absent in

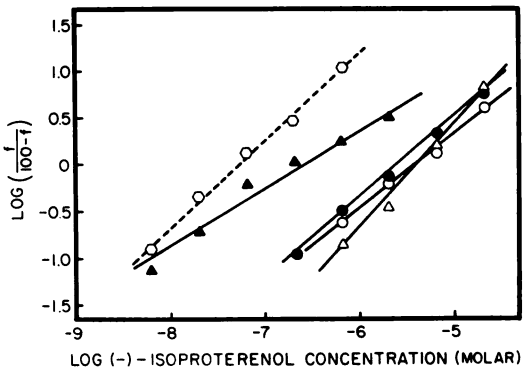

FIG. 6. *Hill plot of (−)-isoproterenol displacement binding to agonist/NEM-sensitive and -resistant receptors in wild-type, unc, and cyc*$^-$ *membranes*

(−)-Isoproterenol displacement data in the absence of NEM for wild-type (▲, from Fig. 4A), *unc* (○, from Fig. 4C), and *cyc*$^-$ (●, from Fig. 4D) membranes are replotted as Hill plots (f is the percentage of specific binding). A Hill plot of (−)-isoproterenol displacement binding to agonist NEM-resistant receptors in wild-type membranes (△) is also shown. It was obtained by preincubation of membranes with 1.67 mM NEM and 33.3 nM (−)-isoproterenol for 15 min at 30°C. These conditions result in the maximal 65% decrease in the number of [^{125}I]IHYP binding sites. The mixture was then diluted 1.67-fold and incubated with 100 pM [^{125}I]IHYP in the presence of the indicated concentrations of (−)-isoproterenol. [^{125}I]IHYP binding in the presence of 20 nM (−)-isoproterenol and 1 mM NEM (final concentrations) represents 100% specific binding to the agonist plus NEM-resistant receptor population (△, see Table 1). Values are means of six determinations. A Hill plot of (−)-isoproterenol displacement binding to agonist plus NEM-sensitive receptors (◯, R_s) in wild-type membranes was obtained by subtracting values for the percentage (−)-isoproterenol binding to the agonist NEM-resistant receptor population (△, R_r) from the percentage (−)-isoproterenol binding to the total population (▲, R_t) by the following equation: $R_s = [100 \times (R_t - 0.35 \times R_r)]/65$. K_D, n_H, and r values for each plot are given in the text. All lines were fitted by a least-squares procedure.

the presence of the antagonists [^{125}I]IHYP (Table 1) and (−)-propranolol (Fig. 1). The same phenomenon was also observed for turkey erythrocyte β-adrenergic receptors, where a linear correlation was found between the rate of inactivation and the intrinsic activity of the adrenergic agent in stimulating the adenylate cyclase (11). A particular feature of the S49 lymphoma cell system resides in the availability of variant cell lines in which catecholamines no longer activate adenylate cyclase (16). The deficiency underlying the functional uncoupling of the β-adrenergic receptor is apparently explained by the absence of a functional guanine nucleotide regulation (coupling) protein in *cyc*$^-$ and the protein's presence in a defective form in *unc* (27). Our data (Figs. 4C and D) show that this nucleotide regulatory protein is also required for β-adrenergic agonists to induce the conformational modification in their receptor necessary for NEM alkylation. The absence of such an interaction between receptor and nucleotide regulatory protein is also sufficient explanation for the previously demonstrated lack of effect of GTP and Mg^{2+} on agonist binding in *unc* and *cyc*$^-$ membranes (7, 17). Further, this conclusion is consistent with the observation that solubilized turkey β-adrenergic receptors no longer stimulate adenylate cyclase activity (20), are not physically associated with the solubilized catalytic subunit and nucleotide regulatory proteins (20, 23), and can no longer be inactivated by NEM in the presence of agonist (Fig. 5). We have no data at present that indicate whether or not this β-agonist-induced conformational change in the receptor also requires the catalytic subunit or possibly an as yet unknown, additional protein in the receptor–cyclase complex (28) as we have suggested to be involved in hormone-sensitive Mg^{2+} transport. It can be argued that the effect of NEM is not on the receptor but on the nucleotide coupling protein. Our preliminary experiments with NEM and prostaglandin E_1 show that S49 membrane incubation with these agents followed by washing does not alter the density of β-adrenergic receptors in a subsequent binding assay, suggesting that β-receptor interaction with the nucleotide coupling protein is normal.

Conversely, the data also suggest that for structural or stoichiometric reasons, the agonist/NEM-resistant receptor subpopulation is not functionally coupled to the cyclase system. This is indicated by (a) the lack of an agonist/NEM effect in preparations in which the receptor is functionally uncoupled from cyclase catalytic activity, (b) the similarity of the binding characteristics of agonist/NEM-resistant receptors in wild-type cells to the functionally uncoupled receptors in *unc* and *cyc*, and (c) the close similarity of the binding characteristics of agonist/NEM-sensitive receptors to those of the receptors responsible for agonist activation of adenylate cyclase activity. For example, (−)-isoproterenol has a K_D of 2 μM and n_H of 1.1 for agonist/NEM-resistant receptors and a K_D of 1–2 μM and n_H of 0.9 for uncoupled receptors in *unc*$^-$ and *cyc*$^-$. In contrast, agonist/NEM-sensitive receptors have a K_D of 30 nM and n_H of 1.0 in the presence of Mg^{2+} (Fig. 6), while they have, by extrapolation, a K_D of about 1–2 μM and n_H of 0.9 in the absence of Mg^{2+} or in the presence of both Mg^{2+} and GTP (see Ref. 7). Those receptors which activate adenylate cyclase in S49 have a K_D of 30 nM and n_H of 1.0. Thus only those receptors sensitive to agonist/NEM have binding parameters which match those of the receptors that activate adenylate cyclase. Binding parameters for neither the total receptor population nor the agonist/NEM-resistant population match those of the receptors which activate the cyclase. This is a correlation, however, and the roles of GTP and free Mg^{2+} are not yet completely defined. Nevertheless, the data herein and those previously reported for S49 cells (7, 17, 29) indicate that changes in β-adrenergic receptor conformation induced by agonist, GTP, Mg^{2+}, or combinations thereof and measured by agonist affinity or NEM alkylation absolutely require interaction between the receptor and the nucleotide regulatory protein.

Our data with the β_d variant of S49 also provide some insight into the nature of the two receptor subpopulations. The partial inactivation of receptor in S49 and presumably other systems by agonist/NEM could be due to structural or stoichiometric limitations of receptor–cyclase coupling or to the existence of two genetically and molecularly distinct subpopulations of the β-adrenergic receptor in a single cell, both having β_2 pharmacologic specificity. The latter explanation is less likely since the reduction of total receptor density in the β_d clone seems to be comprised of an equiproportional reduction in both the coupled and the uncoupled receptor populations. This conclusion is based on both the proportionality between adenylate cyclase activation and β-adrenergic receptor density in wild-type and various β_d clones (26) and the similar ratios of agonist/NEM-sensitive to agonist/NEM-resistant receptors in wild-type and β_d membranes. The data thus suggest that the two subclasses of receptor are not genetically distinct molecules and that the basis for the differential sensitivity to agonist/NEM and the disclosed receptor heterogeneity does not lie in the receptor molecule itself but in structural or stoichiometric limitations of receptor–cyclase coupling.

The present study clearly suggests that only a fraction of the β-adrenergic receptors in a cell can be coupled to the adenylate cyclase complex in the presence of agonist molecules. This fact results in a functional heterogeneity of the receptors under certain experimental circumstances, and thus must be taken in consideration when determining agonist affinities, cooperativity, or other thermodynamic parameters of the total receptor population.

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