Interaction between β-adrenergic receptors and guanine nucleotide sites in turkey erythrocyte membranes

[(-)-[3H]dihydroalprenolol binding/receptor conformation/N-ethylmaleimide/5'-guanylyl imidodiphosphate]

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ABSTRACT β-Adrenergic receptors from turkey erythrocyte membranes have been identified by specific binding of the radiolabeled antagonist (-)[3H]dihydroalprenolol. These receptors are inactivated by the alkylating agent N-ethylmaleimide when occupied by β-adrenergic agonists but not when occupied by antagonists or when unoccupied. A time-dependent decrease of the number of receptor sites is observed. Inactivation affects 45–80% of the sites, regardless of the agonist or N-ethylmaleimide concentration. The guanine nucleotides GTP and 5'-guanylylimidodiphosphate effectively protect the receptors against agonist-mediated inactivation by N-ethylmaleimide. Protection by ATP necessitates a 100-fold higher concentration; 10 mM NaF is ineffective. The guanine nucleotide effect is reversible and occurs via interaction with N-ethylmaleimide-insensitive sites. These observations establish that guanine nucleotide sites interact with and cause structural modification of the agonist-occupied β-adrenergic receptors in turkey erythrocyte membranes.

The β-adrenergic receptor and the adenylyl cyclase enzyme are distinct components of the plasma membrane, separable from each other by gel filtration (1) and affinity chromatography (2). Additional evidence suggests the existence of a third component through which guanine nucleotides allow activation of adenylyl cyclase. These guanine nucleotide binding sites are distinct and separable from both the receptor and the adenylyl cyclase catalytic unit (3–5). In addition, guanine nucleotides regulate β-adrenergic receptors: GTP and analogs such as 5'-guanylylimidodiphosphate (p[NH]ppG) decrease the binding affinity of agonists for the β-adrenergic receptors in glioma and lymphoma cells (6, 7) and frog erythrocytes (8). The agonist-induced desensitization of the frog erythrocyte β-adrenergic receptors can readily be reversed by addition of guanine nucleotides (9), via a shift from a high-affinity catecholamine–receptor complex to a low-affinity rapidly dissociable complex (10).

In contrast to these observations, guanine nucleotide regulation of turkey erythrocyte β-adrenergic receptors cannot be demonstrated by the above procedures. GTP and p[NH]ppG do not affect the affinity of the β-adrenergic agonists for binding to the receptors unless the membranes are pretreated with agonists plus GMP (11), nor does desensitization of the β-adrenergic receptors occur upon prolonged exposure to catecholamines (12, 13). However, modulation of turkey erythrocyte β-adrenergic receptors by guanine nucleotides can be directly demonstrated by the use of an alternative approach. We have shown (13, 14) that β-adrenergic agonists cause a conformational change in 50% of the (-)[3H]dihydroalprenolol binding sites.

This phenomenon was evidenced by the susceptibility of agonist receptor complexes to inactivation by the alkylating agent N-ethylmaleimide, which has no effect on the free or antagonist-bound forms of the receptor. We now show that the guanine nucleotides prevent inactivation of the agonist–receptor complexes by N-ethylmaleimide.

EXPERIMENTAL PROCEDURES

Materials. The following were obtained as gifts: (-)-isoproterenol bitartrate and (-)-epinephrine bitartrate from Sterling Winthrop, and (±)-alprenolol hydrochloride from Ciba-Geigy. N-Ethylmaleimide was purchased from Fluka, GTP, p[NH]ppG, and ATP were from Boehringer Mannheim, and (-)[3H]dihydroalprenolol hydrochloride (3 Ci/mmol; 1 Ci = 3.7 x 10¹⁰ becquerels) was obtained from New England Nuclear. All other reagents were of analytical grade.

Turkey erythrocyte membranes were prepared according to Øye and Sutherland (15), with modifications (16). Membranes were stored in 10 mM Tris-HCl, pH 7.4/145 mM NaCl/2 mM MgCl₂/10% (vol/vol) glycerol at a concentration of 10–15 mg of protein per ml in liquid nitrogen up to 2 months, without loss in (-)[3H]dihydroalprenolol binding, adenylyl cyclase activity, or susceptibility of the binding sites to N-ethylmaleimide in the presence of β-adrenergic agonists.

Preincubation of the Membranes. Membranes (2 mg of protein per ml) were preincubated with the indicated compounds for various periods of time at 30°C in 75 mM Tris-HCl, pH 7.4/25 mM MgCl₂ in a total volume of 1 ml. When the medium contained N-ethylmaleimide (50 μM) and (-)-isoproterenol (50 nM), preincubation was terminated by addition of 100 μl of 20 mM cysteine, and the reaction mixture was centrifuged for 1 min in an Eppendorf micro centrifuge. The precipitated membranes were resuspended in 1 ml of the same buffer and washed twice more.

Assays. For measurement of binding of (-)[3H]dihydroalprenolol, after preincubation and washing, membranes (2 mg/ml) were incubated with 10 nM (-)[3H]dihydroalprenolol in 75 mM Tris-HCl, pH 7.4/25 mM MgCl₂ for 10 min at 30°C. Bound (-)[3H]dihydroalprenolol was measured by filtration on glass fiber filters (Whatman GF/F) as described (2). Non-specific binding was defined as the (-)[3H]dihydroalprenolol bound in the presence of 1 μM (±)-alprenolol and was subtracted from total binding to yield "specific binding." Results presented in all figures and tables represent specific binding obtained by three determinations from two separate experiments and agreeing within 5%.

Protein concentration was measured by the method of Lowry et al. (17), with bovine serum albumin as the standard, and adenylyl cyclase was assayed as described (2).


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RESULTS

The specific (-)-[^3]H]dihydroalprenol binding sites on turkey erythrocyte membranes have been identified as β-adrenergic receptors (2). Binding occurs to a single class of noncooperative sites with an equilibrium dissociation constant \( K_d \) of 8 ± 2 X 10^{-9} M; it shows rapid association/dissociation kinetics (16).

Pretreatment of the membranes with either 50 μM N-ethylmaleimide or 50 nM (-)-isoproterenol for 15 min at 30°C did not affect subsequent binding of 10 nM (-)-[^3]H]dihydroalprenolol. However, pretreatment with both compounds in combination gave rise to a time-dependent fall in the binding activity (Fig. 1). This fall reflects a decrease in the number of receptor sites. Because the affinity of (-)-[^3]H]dihydroalprenolol for the remaining sites was not affected (Fig. 2), the number of receptor sites could be monitored by binding of a subsaturation concentration of (-)-[^3]H]dihydroalprenolol (10 nM).

In a previous report (14), it was shown that this inactivation process affects 45–60% of the receptor sites, depending on the membrane preparation, regardless of the agonist and N-ethylmaleimide concentrations. Approximately 50% of the receptor sites could be inactivated in the membrane preparations used for the present study. When calculated as a function of the amount of sensitive sites (B), the rate of inactivation can be obtained from the data presented in Fig. 1. The linear relationship between in B and the preincubation time (t) is indicative for a pseudo-first-order reaction (Fig. 1 Inset). The apparent first-order rate constant \( k_{ab} \), defined by the absolute value of the semilogarithmic plot, is 0.14 ± 0.02 M^{-1} min^{-1} (from four determinations).

![FIG. 1. Inactivation of (-)-[^3]H]dihydroalprenolol (DHA) binding sites by (-)-isoproterenol plus N-ethylmaleimide: Effect of NaF and p[NH]ppG. Membranes were pretreated with 50 nM (-)-isoproterenol and 50 μM N-ethylmaleimide only (•) or with addition of 10 mM NaF (X) or 50 μM p[NH]ppG (○) for the indicated periods of time at 30°C, washed, and incubated with 10 nM (-)-[^3]H]dihydroalprenolol. Control binding refers to binding of (-)-[^3]H]dihydroalprenolol to membranes pretreated with (-)-isoproterenol plus N-ethylmaleimide for less than 10 sec (i.e., 0.11 pmol/mg of protein). (Inset) Semilogarithmic representation of the same binding data. Inactivation affected 50% of the binding to the inactivable sites [i.e., B = 100 × (observed binding − resistant binding)/total binding] after pretreatment for time t. The pseudo-first-order rate constant for the inactivation process \( k_{ob} \) is defined by the absolute value of the slope of the semilogarithmic plot [i.e., \( \ln (B/B_0) = -k_{ob}·t \), with \( B_0 \) for \( t = 0 \) and equals 0.17, 0.12, or 0.0002 M^{-1} min^{-1} for preincubation with (-)-isoproterenol plus N-ethylmaleimide alone or in the presence of NaF or p[NH]ppG, respectively.](image-url)

![FIG. 2. (-)-[^3]H]Dihydroalprenolol (DHA) binding characteristics after treatment of the membranes with (-)-isoproterenol plus N-ethylmaleimide, in presence or absence of p[NH]ppG. Scatchard plot of (-)-[^3]H]dihydroalprenolol saturation binding (1–50 nM) to erythrocyte membranes. Membranes were pretreated for 15 min at 30°C with buffer only (●) or with 50 nM (-)-isoproterenol plus 50 μM N-ethylmaleimide in the absence (○) or presence (●) of 50 μM p[NH]ppG and washed. The total amount of binding sites \( (B_{max}) \) and the equilibrium dissociation constant for binding \( (K_d) \) for each membrane sample were: ○, \( K_d = 7.3 × 10^{-9} \); \( B_{max} = 0.290 \) pmol/mg of protein; ○, 7.4 × 10^{-9}, 0.153; ●, 7.2 × 10^{-9}, 0.300.](image-url)

Influence of NaF and p[NH]ppG. As shown in Fig. 1, inactivation of β-adrenergic receptors by (-)-isoproterenol plus N-ethylmaleimide was not affected by addition of 10 mM NaF. The time course \( (k_{ab} = 0.12 \) M^{-1} min^{-1}) and the number of sensitive binding sites remained unchanged. In contrast, addition of 50 μM p[NH]ppG effectively abolished the inactivation process \( (k_{ab} = 0.002 \) M^{-1} min^{-1}) (14). The binding affinities for (-)-[^3]H]dihydroalprenolol \( (K_d = 10 ± 2 × 10^{-9} \) M) and (-)-isoproterenol \( (K_d = 0.07 ± 0.2 × 10^{-6} \) M) were not affected by either p[NH]ppG or NaF (ref. 11; other data). Thus, protection by the nucleotide cannot be explained by a decrease in the fractional occupancy of the receptor sites by the agonist. Protection also was independent of the fractional agonist-bound receptor sites. Preincubation of the membranes with 50 μM N-ethylmaleimide in addition to increasing amounts of (-)-epinephrine caused a dose-dependent increase in rate of inactivation (14) which resulted in decreased (-)-[^3]H]dihydroalprenolol binding activity, up to 50% (Table 1). For all tested concentrations of (-)-epinephrine (0.22–54 μM, causing 10–96% occupation of the receptor sites, respectively), addition of 50 μM p[NH]ppG to the preincubation medium effectively abolished inactivation of the receptor sites.

Both NaF and p[NH]ppG are known to cause persistent activation of adenylate cyclase (18–20). Incubation of the turkey erythrocyte membrane preparation used in Fig. 1 for 20 min at 30°C without or with 10 mM NaF, 50 μM p[NH]ppG, or 50 μM p[NH]ppG plus 50 μM (-)-isoproterenol caused the generation of 0.03, 0.38, 0.29, and 0.95 nmol of cyclic AMP per mg of protein, respectively. Thus, although both NaF and p[NH]ppG caused pronounced and comparable adenylate cyclase activation, only the latter compound was able to abolish
Table 1. Inactivation of (-)-[3H]dihydroalprenolol (DHA) binding sites by N-ethylmaleimide plus increasing concentrations of (-)-epinephrine, in the absence or presence of p[NH]ppG

<table>
<thead>
<tr>
<th>(-)-Epinephrine, µM</th>
<th>Receptor occupancy, %</th>
<th>No addition</th>
<th>50 µM p[NH]ppG</th>
<th>100 µM p[NH]ppG</th>
</tr>
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<tbody>
<tr>
<td>0.22</td>
<td>10</td>
<td>85</td>
<td>105</td>
<td></td>
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<tr>
<td>0.67</td>
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<td>69</td>
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<tr>
<td>2</td>
<td>50</td>
<td>59</td>
<td>98</td>
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<tr>
<td>6.0</td>
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<td>94</td>
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</tr>
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<td>54</td>
<td>96</td>
<td>50</td>
<td>95</td>
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</tbody>
</table>

Membranes were pretreated for 15 min at 30°C with 50 µM N-ethylmaleimide plus the indicated concentration of (-)-epinephrine in the absence or presence of 50 µM p[NH]ppG, washed, and incubated with 10 nM (-)-[3H]dihydroalprenolol. Control binding refers to binding of (-)-[3H]dihydroalprenolol to membranes pretreated with buffer only. Receptor occupancy by the agonist equals 100 × (1 + Kd(Ep)/EP), in which Kd(Ep) represents the equilibrium dissociation constant for (-)-epinephrine binding and equals 2 ± 0.7 x 10^-6 M (14). Kd(Ep) was unaffected by 50 µM p[NH]ppG (data not shown).

Table 2. Inactivation of (-)-[3H]dihydroalprenolol binding sites by (-)-isoproterenol plus N-ethylmaleimide: Effect of prior, simultaneous, or subsequent addition of p[NH]ppG and GTP

<table>
<thead>
<tr>
<th>Membrane treatments</th>
<th>First preincubation</th>
<th>Second preincubation</th>
<th>Inactivation rate (kobs), M^-1-min^-1</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Series A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>-</td>
<td>p[NH]ppG</td>
<td>-</td>
<td>0.00 ± 0.01</td>
</tr>
<tr>
<td>-</td>
<td>GTP</td>
<td>-</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>MalNEt</td>
<td>-</td>
<td>p[NH]ppG</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>MalNEt</td>
<td>p[NH]ppG</td>
<td>GTP</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>IPR + p[NH]ppG</td>
<td>-</td>
<td>-</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>IPR + GTP</td>
<td>-</td>
<td>-</td>
<td>0.15 ± 0.02</td>
</tr>
<tr>
<td>Series B</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>-</td>
<td>p[NH]ppG</td>
<td>-</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>IPR + p[NH]ppG</td>
<td>-</td>
<td>-</td>
<td>0.17 ± 0.02</td>
</tr>
<tr>
<td>IPR + GTP</td>
<td>-</td>
<td>-</td>
<td>0.12 ± 0.01</td>
</tr>
</tbody>
</table>

Membranes were pretreated with buffer only or with the indicated combinations of 50 nM (-)-isoproterenol (IPR), 50 µM or 2 mM N-ethylmaleimide (MalNEt), 50 µM p[NH]ppG, and 50 µM GTP. In series A, membranes were preincubated for a fixed period of time (15 min) at 30°C, followed by washing with buffer only. The second preincubation was conducted in the inactivation medium [(−)-isoproterenol plus N-ethylmaleimide] for various periods of time (0, 3, 6, 10, and 15 min) followed by washing with buffer containing 2 mM cysteine. Binding of 10 nM (-)-[3H]dihydroalprenolol was measured for each of the resulting samples, and the rate of inactivation (kobs) calculated according to the legend of Fig. 1. Values are means ± SEM of two experiments. In series B, the procedure was as in series A except that time-dependent preincubation in the inactivation medium was followed by fixed-time preincubation of each of the samples. The later shows (-)-[3H]dihydroalprenolol binding to membranes after 6-min pretreatment with increasing concentrations of nucleotide in addition to 50 nM (-)-isoproterenol and 50 µM N-

![Fig. 3. Dose-dependent protection of the (-)-[3H]dihydroalprenolol (DHA) binding sites by p[NH]ppG, GTP, and ATP. Membranes were pretreated with 50 nM (-)-isoproterenol and 50 µM N-ethylmaleimide for 6 min at 30°C in the presence of increasing concentrations of p[NH]ppG (•), GTP (○), or ATP (△) and then were washed three times, and binding of 10 nM (-)-[3H]dihydroalprenolol was measured. (Inset) kobs values, determined for each experimental value according to the legend of Fig. 1, are presented as a function of the concentration of added nucleotide.](https://example.com/fig3)
ethylmaleimide. Concentrations of p[NH]ppG and GTP exceeding 0.1 μM caused a dose-related protection of the receptor sites, with full protection at 10 μM. GTP and p[NH]ppG were almost equipotent in preventing the inactivation process; \( k_{ob} \) was decreased by 50% at about 1 μM nucleotide in both cases.

A dose-related protection also was conferred by ATP (Fig. 3), although it was significantly less potent (Fig. 3 Inset). The 100-fold higher potency of GTP and p[NH]ppG indicates that receptor protection is mediated via a guanine nucleotide-specific site in the membrane.

**DISCUSSION**

We have shown (13, 14) that the alkylating agent N-ethylmaleimide causes a time-dependent decrease in the number of turkey erythrocyte \( \beta \)-adrenergic receptors only when the receptors are occupied by \( \beta \)-adrenergic agonists. This inactivation affects 45–60% of the receptor sites [i.e., \((-\)\(^{3}H\)dihydroalprenolol binding sites]. The data presented here indicate that the guanine nucleotides GTP and p[NH]ppG prevent this inactivation in a reversible manner. The site through which this effect occurs is, in its native form, insensitive to N-ethylmaleimide and has a greater affinity for guanine nucleotides than for ATP. These data indicate an interaction between guanine nucleotide sites and \( \beta \)-adrenergic receptors in turkey erythrocyte plasma membranes.

Little is known about the number of components that constitute hormone-responsive adenylate cyclase systems or about their organization in the membrane. A generally accepted model (18) suggests that the catecholamine-stimulated adenylate cyclase system is composed of the \( \beta \)-adrenergic receptor, a guanine nucleotide-dependent coupling factor (4, 5) possessing a GTPase activity in turkey erythrocyte membranes (19, 20), and the adenylate cyclase catalytic unit. According to this model, the guanine nucleotide regulation at the level of the \( \beta \)-adrenergic receptor, presented in this communication, would thus occur via the coupling factor. However, comparison of the characteristics of the guanine nucleotide sites that regulate the receptor with the reported properties of the GTPase suggests that these components are not identical.

(1) The catecholamine-stimulated GTPase (coupling factor) in turkey erythrocyte membranes is, in its native form, sensitive to N-ethylmaleimide (19). Under the same conditions (10-min pretreatment of the membranes with 2 mM N-ethylmaleimide at 30°C), the receptor regulatory site is not (Table 2).

(2) Guanine nucleotide modulation of the affinity of the receptor for agonists, mediated through the coupling factor, is only observed after pretreatment of the membranes by agonist plus GMP (11). However, the protective effect by guanine nucleotides described in this work does not require such agonist plus GMP pretreatment.

(3) A kinetic analysis by Tolkovsky and Levitzki (21) indicates that the agonist-bound receptor causes adenylate cyclase stimulation in turkey erythrocyte membranes through a transient, short-lived encounter with the GTPase–adenylate cyclase complex. The full protection of the receptor by p[NH]ppG and GTP, however, is indicative of a steady interaction between the receptors and their regulatory guanine nucleotide sites.

Although final proof will require purification and characterization of all components involved in catecholamine-stimulated adenylate cyclase, the considerations reported here suggest that those guanine nucleotide sites that affect adenylate cyclase activity (coupling factor) and those that modify the receptor–N-ethylmaleimide interaction may be borne by distinct membrane components. We recently proposed (22) a model that integrates, in the receptor cyclase system, two different guanine nucleotide binding sites, one of which is the coupling factor.

Inactivation of the \((-\)\(^{3}H\)dihydroalprenolol binding sites by \( \beta \)-adrenergic agonists and N-ethylmaleimide has been explained by an agonist-induced change of the receptor conformation, resulting in an enhanced alkylability (13, 14). In this context, the above-mentioned ability of guanine nucleotides to abolish the inactivation process suggests that occupation of the guanine nucleotide sites results in a new conformation of the receptor that is no longer sensitive to alkylation.

At the present level it is not determined whether this guanine nucleotide site is part of the receptor molecule or is borne by a distinct membrane component. Our previous observation (23) that the affinity chromatography purified \( \beta \)-adrenergic receptors do not possess measurable p[NH]ppG binding activity suggests the existence of a distinct nucleotide site.

\( \beta \)-Adrenergic agonists do not cause demonstrable desensitization of the \( \beta \)-adrenergic receptors in turkey erythrocyte membranes (12, 13). Instead, they allow N-ethylmaleimide to cause a time-dependent decrease in the number of \((-\)\(^{3}H\)dihydroalprenolol binding sites. This inactivation process shares some properties with desensitization of \( \beta \)-adrenergic receptors in other systems such as frog erythrocytes (24) and S49 lymphoma cells (25). Both processes are induced only in the presence of agonist molecules and affect about 50% of the receptor sites. Guanine nucleotides prevent inactivation in turkey erythrocyte membranes and cause resensitization of the \( \beta \)-adrenergic receptors in frog erythrocytes (24) in a reversible fashion. The inability of nucleotides to restore receptor activity in turkey erythrocyte membranes is presumably due to the irreversible nature of the alkylation. The fact that desensitization, inactivation, and protection concern 50% of the binding sites suggests that these phenomena affect the same molecules. The remaining receptors, although analogous in binding properties, must either be structurally different or be located in a protective environment.

These similarities suggest that agonist binding to the \( \beta \)-adrenergic receptors triggers the same kind of molecular interactions in turkey erythrocytes as they do in frog erythrocytes and S49 lymphoma cells. The fact that desensitization cannot be demonstrated in the turkey erythrocyte system reflects a more pronounced conformational stability of the \( \beta \)-adrenergic receptor. The protective effect of guanine nucleotides against N-ethylmaleimide-induced modification of the receptor is reversible (Table 2). It is still unclear whether this is due to rapid displacement of bound nucleotide induced by agonist, as was recently proposed (11) for the reversible effect on agonist affinity.

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