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DUAL EFFECT OF *N*-ETHYLMALEIMIDE ON AGONIST-MEDIATED CONFORMATIONAL CHANGES OF β-ADRENERGIC RECEPTORS

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Abstract—β-Adrenergic agonists cause adenylate cyclase activation via a conformational change of their receptor. This was earlier revealed by the use of *N*-ethylmaleimide as a structural probe: agonist-bound receptors are rapidly inactivated by 0.1 mM *N*-ethylmaleimide while free and antagonist-bound receptors remain unaffected. Furthermore, the β-adrenergic receptors only change conformation when coupled to the guanine nucleotide regulatory component of the adenylate cyclase system.

It is shown in this report that treatment of turkey erythrocyte membranes with the sulphydryl-specific agent 2,2′-dinitro-5,5′-dithiodibenzoic acid and elevated concentrations of *N*-ethylmaleimide (1–10 mM) do not affect the total receptor number, but prevent the ability of β-adrenergic agonists to mediate conformational changes. This effect is mimicked by GTP. These three compounds cause also a two- to four-fold decrease in agonist affinity. Both phenomena may be explained by the ability of the reagents to prevent, and of GTP to reverse, the functional coupling between the receptors and the regulatory component of the adenylate cyclase system.

Removal of Mg^{2+} from the incubation medium (i.e. presence of 1 mM EDTA) produces a similar decrease in agonist affinity, but does not impair the ability of agonist/*N*-ethylmaleimide to inactivate the receptors. This suggests that Mg^{2+} increases the agonist affinity for the receptor–regulatory component complex, but is not required for its formation.

Although all β-adrenergic ligands bind to a common receptor specific (−)-[^{3}H]DHA§ binding sites, only agonists induce a sequence of events in the membrane resulting in adenylate cyclase stimulation. We have recently provided a molecular basis for this distinction by showing that agonists but not antagonists mediate a conformational change of a given subpopulation of β-adrenergic receptors: 45–60% in turkey erythrocyte membranes [1], 65% in wild type S49 lymphoma cell membranes [2] and 50% in human adipose membranes [3]. These conformational changes could be monitored by use of NEM as a structural probe: 0.1 mM or less of NEM causes rapid inactivation of agonist-bound receptors, but was ineffective in the absence of ligand or in the presence of antagonist molecules. The rate of receptor inactivation was proportional to the intrinsic activity of the considered agonist [1]. Coupling to the guanine nucleotide regulatory component of the adenylate cyclase system was found to be essential for the receptors to undergo such conformational changes: the receptors were agonist/NEM-resistant after solubilization [2], desensitization [3] and in the S49 *unc* and *cyc*$^-$. variants in which the receptors cannot undergo functional coupling to the regulatory component [2].

Several groups have recently reported that, when used in a higher concentration range (5–10 mM), NEM might have a distinct action on β-adrenergic receptors. Under these conditions, the reagent causes a decrease in agonist affinity of the receptors without affecting the total receptor number in various cell types [4–6] and prevents the ability of agonists to induce receptor desensitization in intact frog erythrocytes [5]. These results were interpreted by the faculty of NEM to prevent functional coupling between the receptors and the guanine nucleotide regulatory components of the adenylate cyclase system.

In this report, we present evidence for this duality of NEM action in the turkey erythrocyte system, by showing that inactivation of agonist-bound receptors by 0.1 mM NEM is impaired after membrane preincubation with 1–10 mM of the reagent. High concentrations of NEM prevent coupling between the receptor and the regulatory component, whereas low concentrations of the reagent can be used in the presence of agonist to cause inactivation of, and thus to evidence the presence of coupled receptors.

MATERIALS AND METHODS

Materials. (−)-Isoproterenol bitartrate was a kind gift from Sterling Winthrop (Rensselaer, NY). (±)-Alprenolol hydrochloride was from Ciba-Geigy (Basle, Switzerland). *N*-Ethylmaleimide (NEM) was purchased from Sigma Chemical Company (St.

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§ Abbreviations: NEM, *N*-ethylmaleimide; DTNB, 2,2′-dinitro-5,5′-dithiodibenzoic acid; (−)-[^{3}H]DHA, (−)-[^{3}H]dihydroalprenolol.

Louis, MO), 2,2-dinitro-5,5′-dithiodibenzoic acid (DTNB) was from Merck (Darmstadt, FRG) and GTP from Boehringer Mannheim (Mannheim, FRG). (−)-[^{3}H]Dihydroalprenolol hydrochloride (33 Ci/mmole) was obtained from New England Nuclear Corporation. All other chemicals were of analytical grade.

Preparation of turkey erythrocyte membranes. Turkey erythrocyte membranes were prepared as earlier described [7] with the following modification: washed cells were lysed in a 10-fold volume of 5 mM Tris–HCl (pH 7.4)/2 mM $MgCl_2$ for 10 min at 4°, and sedimented by centrifugation at 1000 *g* for 15 min. The supernatant was removed by aspiration, and the lysis was repeated twice before homogenization of the cells.

The present membrane preparations show an increased density of β-adrenergic receptors: i.e. 0.8 pmole receptors per mg membrane protein compared with the 0.2–0.3 pmole/mg for the earlier described cruder preparations [1, 7]. The binding affinity of the tracer ($K_D = 8 \pm 2$ nM) and the fraction (50 ± 5%) of agonist-bound receptors that can undergo a conformational change are unaffected by the new preparation. In contrast with cruder membranes, free Mg^{2+} causes a small increase in agonist affinity: K_D values for (−)-isoproterenol are 0.11 and 0.38 μM in the presence of 25 mM Mg^{2+} and 1 mM EDTA, respectively.

Membrane pretreatments. Membranes (2 mg protein/ml) are preincubated with the indicated compounds for various periods of time at 30° in 75 mM Tris–HCl (pH 7.4)/25 mM $MgCl_2$ in a total volume of 1 ml. The mixture was then transferred into a 1.5 ml micro-test tube (Eppendorf) and centrifuged for 1 min at 12,000 rpm in an Eppendorf centrifuge. After removal of the supernatant by aspiration, the preincubated membranes were resuspended into 1 ml of buffer and centrifuged for 1 min. This washing step was repeated two to four times.

Binding of (−)-[^{3}H]dihydroalprenolol. Binding of (−)-[^{3}H]DHA to the pretreated membranes is performed as described elsewhere [1]. In all figures and tables, bound (−)-[^{3}H]DHA refers to specific binding to the β-adrenergic receptors: i.e. binding of 10 nM of tracer which can be displaced by 10 μM of (±)-alprenolol.

RESULTS

β-Adrenergic receptors have been identified in turkey erythrocyte membranes by the specific binding of (−)-[^{3}H]DHA [1]. In the present membrane preparation, binding occurs to a single class of non-cooperative receptor sites (0.8 pmole/mg protein) with an equilibrium dissociation constant (K_D) of 8 ± 2 nM. β-Adrenergic agonists still cause a conformational change of approximately 50% of their receptors, resulting in their rapid inactivation by 0.1 mM NEM (Table 1, Fig. 3).

Pretreatment of these membranes with 50 μM of the sulphydryl group-specific reagent DTNB for 15 min at 30° does not affect the total receptor number (Table 1). However, NEM is no longer able to inactivate agonist-bound receptors (Fig. 1A). The kinetic data presented in Fig. 1A reveal that membrane pretreatment with DTNB produces a time-wise decrease in the agonist/NEM sensitivity of the receptors. When the number of agonist/NEM sensitive sites is plotted on a logarithmic scale, the decrease appears to be linearly related to the time of exposure (Fig. 1B). Accordingly, the protectory action of DTNB can be quantitated by an apparent first-order rate constant (k_p) which is equal to the absolute value of the slope of the semi-logarithmic plot. The rate of receptor protection is dependent on the DTNB concentration. As shown in Fig. 1A and B, k_p increases upon elevation of the DTNB concentration: i.e. $k_p = 0.015$, 0.056 and 0.58 min^{-1} in presence of respectively 5, 10 and 50 μM of the reagent.

Complete protection of the receptors can also be achieved by membrane pretreatment with 10 mM NEM for 15 min at 30° (Fig. 1C). Here again, the total receptor number is not affected (Table 1). The time and NEM-concentration dependency of this protectory action is shown in Fig. 1C and D. Calculated k_p values are 0.007, 0.021, 0.050 and 0.15 min^{-1} in the presence of respectively 0.1, 0.5, 1.0 and 10 mM NEM. Comparison of these data with the k_p values for DTNB reveals that the receptor protecting potency of DTNB is about two orders in magnitude over that of NEM. The protectory action of NEM and DTNB is mimicked by the direct presence of 1 mM GTP in the agonist/NEM inactivation

Table 1. Membranes were preincubated with the indicated compounds, washed, and incubated for 10 min with 10 nM (−)-[^{3}H]DHA

Membrane preincubation (20 min, 30°) with:	(−)-[^{3}H]DHA binding as % of control
IPR (a)	97 ± 4
NEM (10 mM)	100 ± 3
DTNB (1 mM)	95 ± 5
IPR (0.5 μM) + NEM (0.1 mM)	54 ± 4
IPR (0.5 μM) + NEM (0.1 mM) + GTP (1 mM)	98 ± 3
IPR (0.5 μM) + NEM (0.1 mM) + EDTA (1 mM) (b)	49 ± 4

Control binding refers to binding of the tracer to membranes pretreated with buffer only. Values are means ± ranges of two separate experiments.

(a) IPR = (−)-isoproterenol. (b) no free Mg^{2+} was present in the preincubation buffer.

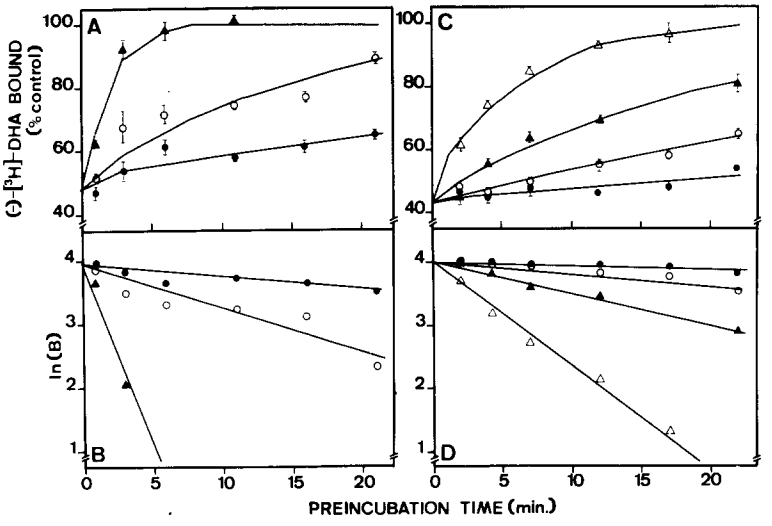

Fig. 1. (A) Turkey erythrocyte membranes (2 mg protein/ml) were preincubated with DTNB (●——●, 5 μM, ○——○, 10 μM, ▲——▲, 50 μM) for the indicated periods of time (abscissa) at 30°, and washed. Complete inactivation of agonist/NEM sensitive β-adrenergic receptors was achieved by subsequent pretreatment of the membranes with 0.1 mM NEM plus 0.5 μM (−)-isoproterenol for 20 min. Membranes were washed again, and the number of remaining receptors was determined by the specific binding of 10 nM (−)-[^{3}H]DHA. Control binding refers to binding of the tracer to membranes pretreated twice with buffer only. Values are means ± ranges of two separate experiments. (B) Semi-logarithmic representation of the same binding data. B represents the number of receptors (as percentage of control) that can still be inactivated by agonist/NEM following membrane pretreatment with DTNB alone. (C) and (D) Experiments are the same as for (A) and (B) except that the first preincubation is performed in the presence of NEM (●——●, 0.1 mM, ○——○, 0.5 mM, ▲——▲, 1 mM, △——△, 10 mM) instead of DTNB.

medium (Table 1). This GTP protection has also been observed by use of cruder membrane preparations [8].

In addition, these three compounds produce a slight, but consistent decrease in the affinity of β-adrenergic agonists for binding to the receptor. As shown in Fig. 2 and Table 2, preincubation of the membranes with 10 mM NEM and 1 mM DTNB (for 15 min at 30°) as well as direct incubation with 1 mM GTP causes a two- to three-fold decrease in affinity of the β-adrenergic agonist (−)-isoproterenol. Binding of the tracer is not affected. The present conditions provide both full protection of the receptors against agonist/NEM and a maximal decrease in agonist affinity (Fig. 1) [8]. At lower reagent concentrations, the decrease in agonist affinity is DTNB

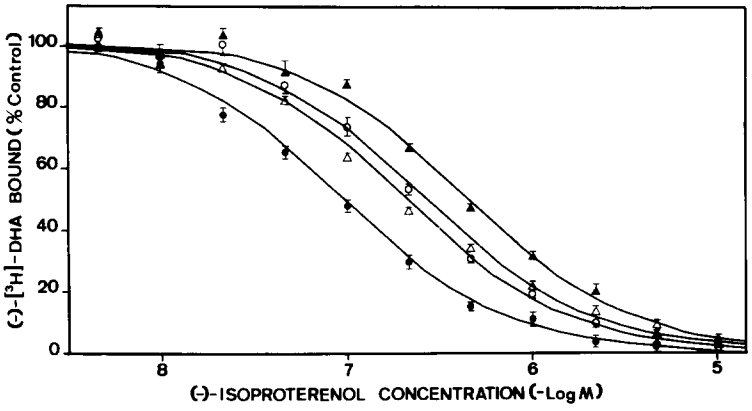

Fig. 2. Membranes are incubated with 10 nM (−)-[^{3}H]DHA in the presence of increasing concentrations of (−)-isoproterenol (abscissa) for 10 min at 30°, after which binding is measured. Control binding refers to binding in the absence of competitor. ●——●, ○——○: Binding to native membranes in the absence and presence of 1 mM GTP, respectively. ▲——▲, △——△: Binding to membranes pretreated for 20 min with 10 mM NEM and 1 mM DTNB, respectively. Values are means ± S.D. of three separate experiments.

Table 2. Membranes were preincubated with the indicated compounds, washed, and incubated for 10 min with 10 nM (−)-[^{3}H]DHA in presence of increasing concentrations of (−)-isoproterenol (as shown in Fig. 2) in the absence or presence of GTP or EDTA

Membrane preincubation (20 min, 30°) with:	K_D for (−)-isoproterenol (in 10^{-7} M)
Buffer only	1.1 ± 0.2
NEM (0.05 mM)	1.2 ± 0.3
NEM (1 mM)	1.4 ± 0.5
NEM (10 mM)	5.1 ± 0.5
DTNB (0.05 mM)	1.1 ± 0.1
DTNB (1 mM)	2.3 ± 0.2
GTP (1 mM)	2.7 ± 0.5
GTP (1 mM) + NEM (10 mM)	4.9 ± 0.2
GTP (1 mM) + DTNB (1 mM)	2.9 ± 0.2
EDTA (1 mM)	3.8 ± 0.7
EDTA (1 mM) + GTP (1 mM)	4.0 ± 0.5

K_D-Values for the agonist are calculated by the method of Cheng and Prusoff [17] from the concentrations causing 50% displacement of bound (−)-[^{3}H]DHA (K_D = 9 nM). Control binding refers to binding of the tracer to membranes pretreated with buffer only. Values are means ± S.D. of three separate experiments.

and NEM dose-dependent, DTNB being more potent than NEM (Table 2). This order of potencies is the same as the one earlier observed for the receptor protectory action of the reagents (Fig. 1).

In a previous report [1], we have demonstrated that the rate of receptor inactivation by agonist/NEM is proportional to the concentration of both the reagent and the agonist–receptor complex. Accordingly, the observed protection by GTP, NEM and DTNB might be the consequence of their ability to lower the agonist affinity of the receptors, and thus the rate of receptor inactivation, rather than the transformation of agonist/NEM sensitive into resistant receptors. To test this eventuality, we have investigated (−)-isoproterenol/NEM inactivation kinetics after pretreatment of the membranes with buffer only or with 10 mM NEM for various periods of time. In Fig. 3 it is clearly shown that the agonist/NEM inactivation has reached completion after 5 min, regardless of the NEM pretreatment conditions, and that this NEM pretreatment causes a concentration-dependent decline in the number of agonist/NEM sensitive receptors. The same picture is obtained after DTNB pretreatment of the membranes (data not shown). Since all other agonist/NEM inactivation experiments are performed for 20 min in this study, the observed receptor protections must thus be related to the tranformation of agonist/NEM-sensitive into resistant receptors, and not to a decline in the inactivation rate.

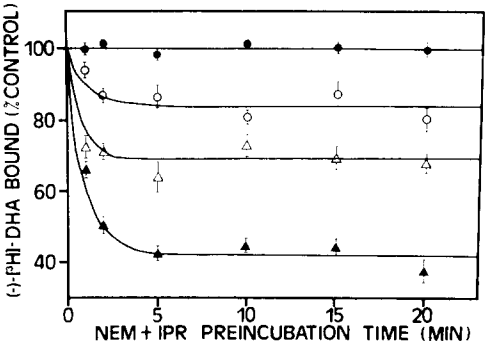

Fig. 3. Membranes (2 mg protein/ml) were preincubated with 10 mM NEM for 1 (▲——▲), 6 (△——△), 10 (○——○) and 20 (●——●) min at 30°, and washed. Inactivation of agonist-bound receptors was achieved by subsequent pretreatment of the membranes with 0.1 mM NEM plus 0.5 μM (−)-isoproterenol for increasing periods of time (0–20 min) at 30°. Membranes were washed again, and the number of remaining receptors was determined by the specific binding of 10 nM (−)-[^{3}H]DHA. Control binding refers to binding of the tracer to membranes pretreated twice with buffer only. Values are means ± ranges of two separate experiments.

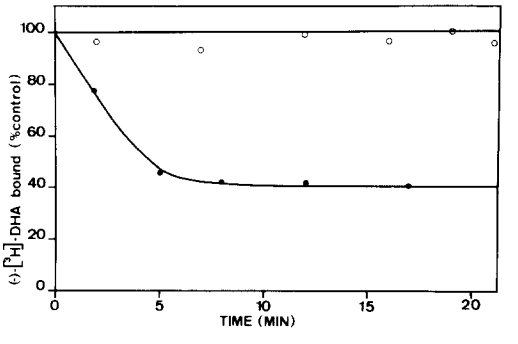

Fig. 4. Membranes were pretreated in 75 mM Tris–HCl (pH 7.4)/1 mM EDTA with 0.1 mM NEM plus 0.5 μM (−)-isoproterenol for increasing periods of time (0–20 min) at 30° either in the absence (●——●) or presence (○——○) of 1 mM GTP. Membranes were then washed and the number of remaining receptors was determined by the specific binding of 10 nM (−)-[^{3}H]DHA. Control binding refers to binding of the tracer to membranes pretreated with buffer only. Values are means ± ranges of two separate experiments.

Removal of Mg^{2+} from the incubation medium (i.e. by replacement of 25 mM Mg^{2+} by 1 mM EDTA) results also in a three-fold drop in the (−)-isoproterenol affinity in the present membrane preparation (Table 2). However, unlike NEM, DTNB and GTP, there is no EDTA protection of the receptors against agonist/NEM inactivation (Fig. 4). Moreover, whereas 1 mM GTP causes no further decrease in the agonist affinity in the presence of EDTA (Table 2), the nucleotide is still able to confer full receptor protection against agonist/NEM (Fig. 4). These data clearly show that a decrease in agonist affinity may coincide with, but is not causually related to receptor protection, and hence that different mechanisms must underlie both phenomena.

DISCUSSION

The data presented in this report suggest that the alkylating agent NEM has two distinct effects on turkey erythrocyte β-adrenergic receptors.

(1) NEM (0.1 mM) causes a rapid inactivation of approximately 50% of the receptors in the presence of β-adrenergic agonists. This process was previously explained by the ability of agonists to mediate conformational changes of the receptors (i.e. from a non-active to an active conformation), resulting in the unmasking of essential alkylable groups at their surface [1].

(2) Pretreatment of the membranes with higher concentrations of NEM (1–10 mM) effectively protects the β-adrenergic receptors against subsequent agonist/NEM inactivation. This phenomenon may be adequately explained by the ability of NEM to impair the functional coupling between the receptors and the guanine nucleotide regulatory component of the adenylate cyclase system. This interpretation is based on the following considerations. (i) Coupling to the regulatory component is necessary for β-adrenergic agonists to induce a conformational modification of the receptor: there is no agonist/NEM effect in preparations in which the receptor is functionally uncoupled such as in solubilized turkey erythrocyte membranes and in membranes from the S49 lymphoma *unc* and *cyc*$^-$ mutants [2]. (ii) NEM provokes uncoupling: this results in a decrease in the agonist affinity in the membranes from several cell types [4–6] including the turkey erythrocyte system (Fig. 2, Table 2) and in the prevention of agonist-mediated desensitization of the receptors in frog erythrocytes [4].

Both NEM actions show a different dependence on the NEM concentration as well as on the presence of agonist. On basis of these differences, they can be investigated separately.

(1) Full receptor protection by NEM is invariably achieved when the membranes are pretreated in the absence of agonist for 20 min at 30° with 10 mM NEM or more (Table 1).

(2) On the other hand, NEM inactivates 50% of the β-adrenergic receptors only in the presence of agonists. This process can be shown to reach completion within 20 min in the presence of 0.1 mM NEM, provided that the agonist concentration equals or exceeds its K_D value for the receptor. Under these conditions, only minimal interference of NEM protection has to be expected since the rate of protection (0.007 min^{-1}, Fig. 1) is much lower than the rate of inactivation (0.27 min^{-1} in the presence of 0.5 μM (−)-isoproterenol, data not shown).

The ability of NEM to confer receptor protection and to decrease the agonist affinity is also shared by the sulphydryl group-specific agent DTNB and by GTP.

First, this indicates that both NEM and DTNB hamper the formation of the receptor–regulatory protein complex. This might be the result of alkylation of free sulphydryl groups present at the surface of proteins governing the membrane structure (putatively affecting the receptor–regulatory protein complex formation), or more specifically, of the receptor of the regulatory protein themselves. Being alkylated by both reagents, the adenylate cyclase enzyme might be a good target for the NEM and DTNB action. This hypothesis is, however, weakened by our observation that preincubation of the membranes with low NEM concentrations (12 min, 0.1 mM NEM, 30°) causes a full drop in subsequent (−)-isoproterenol (10 μM), Gpp(NH)p (0.1 mM) and NaF (10 mM) stimulation of the enzyme (unpublished observations), whereas the same pretreatment does not cause an appreciable decline in the receptor–regulatory protein complex formation (Fig. 1). In addition, recent reconstitution experiments by Jeffery *et al.* [6], suggest that receptor–regulatory protein coupling is not prevented by alkylation of the receptor itself, since turkey erythrocyte receptors show a lower agonist affinity when incorporated in NEM-treated vs native human erythrocyte membranes. Accordingly, NEM and DTNB protection may best be explained by alkylation of the regulatory protein itself, or of yet unidentified membrane proteins which are essential for the coupling process.

Second, the similarity between the reagent and GTP action, together with findings from other laboratories [11, 12] support the hypothesis that the nucleotide provokes uncoupling of the receptor–regulatory protein complex. Several groups have demonstrated that the decrease in agonist affinity by GTP is in fact due to the reversal of a Mg^{2+}-mediated increase in agonist affinity for the receptor–regulatory protein complex [13, 14]. Interestingly, NEM, DTNB and GTP appear to cause parallel leftward shifts of the (−)-isoproterenol competition binding curves in the presence of Mg^{2+} (Fig. 2), suggesting that, in the turkey erythrocyte, both the agonist/NEM sensitive and resistant receptor populations might undergo coupling to metal/guanine nucleotide proteins. However, either the coupling phenomenon, or the metal/guanine nucleotide proteins might be different, since GTP causes a non-competitive decline in Mg^{2+} effect for the agonist/NEM-sensitive receptor population, and a competitive decline for the resistant population (G. Vauquelin *et al.*, manuscript in preparation).

The ability of GTP to decrease agonist affinity in turkey erythrocyte membranes appears to be quite variable from one report to another [8, 15, 16]. Our data reveal that this variability might be the consequence of differences in susceptibility of Mg^{2+} to induce the high agonist affinity state of the complex,

rather than of differences in ability of GTP to provoke uncoupling.

(1) The metal chelating agent EDTA decreases the agonist affinity, but does not protect the receptor against agonist/NEM inactivation (Table 2, Fig. 4). The receptor–regulatory protein complex can thus adopt a low agonist affinity state. This suggests that Mg^{2+} increases the agonist affinity for the receptor–regulatory protein complex but is not required for its formation.

(2) GTP protection was previously shown to occur in less purified membranes, wherein no decrease in agonist affinity was apparent [8]. The ability of Mg^{2+} to increase the agonist affinity might thus be function of the membrane preparation.

The Mg^{2+}, reagent and GTP effects can be integrated in the following model:

$$H + R + G \leftrightharpoons H.R.$$
$$+ G \underset{\text{NEM}}{\overset{}{\underset{\text{DTNB}}{\nleftrightarrow}}} H.R.G \xrightarrow{\text{GTP}} H.R + G.GTP$$

The agonist, receptor and regulatory protein are represented by H, R and G, and their complexes by H.R and H.R.G. The following mechanisms are schematized in this model. (1) H.R but not R alone couples to G. (2) Coupling is prevented by the chemical modification of cysteinyl residues of G or of membrane proteins affecting H.R.G formation. (3) R is in the active conformation (i.e. inactivable by NEM) in the H.R.G complex but neither alone or in the H.R complex. (4) Free Mg^{2+} is able to increase the agonist affinity for the H.R.G complex, but is not required for its formation. (5) GTP uncouples G from H.R, thereby protecting the receptors against agonist/NEM inactivation and decreasing the agonist affinity (in presence of Mg^{2+}).

We have previously suggested that receptor protection against agonist/NEM and decrease in agonist affinity might result from GTP interaction with distinct regulatory proteins [8]. The above model makes it now possible to explain both phenomena on the basis of receptor and GTP interaction with a single regulatory component. This model also stresses the essential role of this regulatory component in the ability of NEM to provoke both receptor protection in the absence of agonist and receptor inactivation in the presence of agonist.

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