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AGONIST/*N*-ETHYLMALEIMIDE MEDIATED INACTIVATION OF β-ADRENERGIC RECEPTORS

MOLECULAR ASPECTS

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Abstract—Incubation of turkey erythrocyte and rat lung membranes with the β-adrenergic agonist isoproterenol in the presence of the group specific reagent *N*-ethylmaleimide (NEM) causes the apparent inactivation of a well defined fraction of the β-adrenergic receptors. This inactivation process requires the coupling between the receptors and the guanine nucleotide binding regulatory component of the adenylate cyclase system. Accordingly, only the isoproterenol/NEM sensitive receptor population is susceptible to undergo this functional coupling. Incubation of the treated membranes in alkaline buffer and/or in the presence of GTP reactivates the receptors. The reactivated receptors show the initial binding properties for both isoproterenol and the radiolabelled antagonist [^{3}H]-dihydroalprenolol and are still dithiothreitol sensitive. Moreover the reactivated receptors can again couple to the regulator in the presence of agonists. The present study makes it possible to discriminate between the different models advanced for the *N*-ethylmaleimide action and is valid for β_1- as well as β_2-adrenergic receptors. The reagent appears indeed to freeze the hormone–receptor–regulator complex by alkylating the latter component, as previously proposed by Korner *et al.* (1982) *J. biol. Chem.* **257**, 3389–3396. It is also suggested that the existence of a limited fraction of coupling-prone receptors results from differences in the membrane microenvironment or from a structural heterogeneity of the receptors rather than from a stoichiometric limitation of the regulator concentration.

It is well known that the agonist-bound β-adrenergic receptor (H.R)* interacts with a guanine nucleotide regulatory protein (Ns) in the pathway of adenylate cyclase activation [1–4]. However, in every membrane preparation studied so far, there is always a proportion of the receptors that is unable to form the ternary H.R.Ns complex.

As a well documented example of this functional receptor heterogeneity, free Mg^{2+} ions increase the agonist affinity for only part of the receptors [5, 6]. This Mg^{2+} effect requires H.R–Ns coupling since it is reversed by guanine nucleotides, which cause the dissociation of Ns from H.R, and is absent in S_{49} lymphoma cell lines wherein Ns is defective or absent [7–9].

The variations of the agonist affinity also reflect conformational changes associated specifically with the H.R.Ns complex formation. This is demonstrated by the fact that, in the presence of agonists, the alkylating reagent *N*-ethylmaleimide (NEM) impairs radioligand binding to a well defined β-receptor population in a variety of tissues and cell types [10–16]. The agonist/NEM sensitive receptor population is the same as that which acquires high affinity for agonists in the presence of Mg^{2+} and the agonist/NEM reaction is prevented in all the conditions known to impair H.R–Ns coupling. Accordingly, both the Mg^{2+} effect and the agonist/NEM effect indicate that only a fraction of the receptors can participate in complex formation with Ns.

Whereas the agonist/NEM reaction is an easy tool for the investigation of the functional heterogeneity of β-adrenergic receptors, it is still not clear whether the reagent attacks R or Ns. It was first suggested that the β-receptor possesses a hidden sulphydryl group which becomes exposed as a result of the formation of the H.R.Ns complex. NEM would then alkylate this sulphydryl group and thereby hamper subsequent radioligand binding to the receptor. Heidenreich *et al.* [11] adopted this hypothesis in part, but suggested moreover that the formation of the H.R.Ns complex would go along with the reduction of a disulfide bond present at the receptor binding site. By alkylating the freed-SH groups, NEM should "freeze" the receptors in a slow agonist dissociation conformation and hence prevent the subsequent radioligand assay of the receptor. The NEM-mediated formation of a slow-dissociating agonist–receptor complex was confirmed by Korner *et al.* [15]. These authors, however, proposed that NEM traps the H.R.Ns complex in a locked conformation by alkylating —SH group(s) presumably located on Ns and not on the receptor itself.

The present study aims at obtaining a better insight in the agonist/NEM action. We present evidence that the agonist/NEM reaction does not cause steric hindrance and does not affect the disulfide bond at the ligand binding site of the receptor. Our ability to obtain functional receptor reactivation (i.e. able to form again a H.R.Ns complex) of agonist/NEM treated membranes, further indicates that the receptor itself has not been alkylated.

* Abbreviations used: NEM: *N*-ethylmaleimide; DTT: dithiothreitol; [^{3}H]-DHA: (−)-[^{3}H] dihydroalprenolol; H, R, G: the β-adrenergic agonist, the receptor, the guanine nucleotide binding regulatory component of the adenylate cyclase system.

MATERIALS AND METHODS

Materials The following materials were used: (−)-Alprenolol hydrochloride, donated by Ciba-Geigy (Basel, Switzerland), (−)-Isoproterenol-(+)-bitartrate and *N*-ethylmaleimide (NEM) purchased from Sigma Chemical Company (St. Louis, MO), 1,4-Dithiothreitol (DTT) from Merck (Darmstadt, F.R.G.) and GTP from Boehringer Mannheim (Mannheim, F.R.G.) (−)-[^{3}H]-Dihydroalprenolol hydrochloride ([^{3}H]-DHA) (92 Ci/mmole), from the New England Nuclear Corporation. All other chemicals were of analytical grade.

Membrane preparations. Turkey erythrocyte ghosts were prepared as described elsewhere [17].

Rat lung membranes preparation: Sprague–Dawley rats (150–250 g) were killed by decapitation and the lungs were rapidly removed. The lung cortex was homogenized in 20 vols of 145 mM NaCl, using first a Ultraturax and then a glass–Teflon homogenizer. The homogenated supernatants were submitted to subsequent differential centrifugation at 900 *g*, at 10,000 *g* and at 40,000 *g*, for 15 min each time. The final pellet was resuspended in 5 mM Tris–HCl (pH 7.4) containing 15% glycerol, and was kept frozen in liquid nitrogen until further use.

Protein determinations were carried out by the method of Lowry *et al.* [16] using bovine serum albumin as the standard.

Membrane pretreatment with isoproterenol/NEM and receptor reactivation. Membranes (a final protein concentration of 2 mg/ml) are preincubated with 0.2 μM isoproterenol and 0.2 mM NEM in 75 mM Tris–HCl (pH 7.6), 25 mM $MgCl_2$ for 10 min at 30°. This pretreatment step was performed in 1.5 ml Eppendorf microtest tubes. Preincubation was followed by centrifugation of the membranes for 2 min in an Eppendorf centrifuge (12,000 rpm) at room temperature. After removal of the supernatant, the precipitated membranes were resuspended in 1 ml buffer. This washing step was repeated twice.

Reactivation of the isoproterenol/NEM treated membranes occurred by 30 min incubation in conditions as indicated in the text.

DTT pretreatment: Membranes were incubated with 1 mM DTT for 10 min at 30° prior to the radioligand binding assay.

Binding of [^{3}H]-DHA. Binding of [^{3}H]-DHA (10 nM on turkey erythrocyte membranes and 5 nM on rat lung membranes) is performed as described elsewhere [17–19]. In all figures and tables, bound [^{3}H]-DHA refers to the specific binding to the β-adrenergic receptors, i.e. binding of tracer which can be displaced by 10 μM of (±)-alprenolol.

Competition curves of agonist displacement of [^{3}H]-DHA binding were computer fitted using an iterative curve fitting program derived from Minneman's [20].

Most experiments were run at least 3 times or more. Bars represent standard deviation.

RESULTS

β-adrenergic receptors were identified in turkey erythrocyte (β_1-) and rat lung membranes (β_2- in excess over β_1-) by the specific binding of the radiolabelled antagonist [^{3}H]-DHA.

When turkey erythrocyte and rat lung membranes are treated with 0.2 mM NEM and 0.2 μM isoproterenol for 10 min, there is a decrease in the number of receptor sites of 55% and 50% respectively (Fig. 1). The affinity of the tracer for the

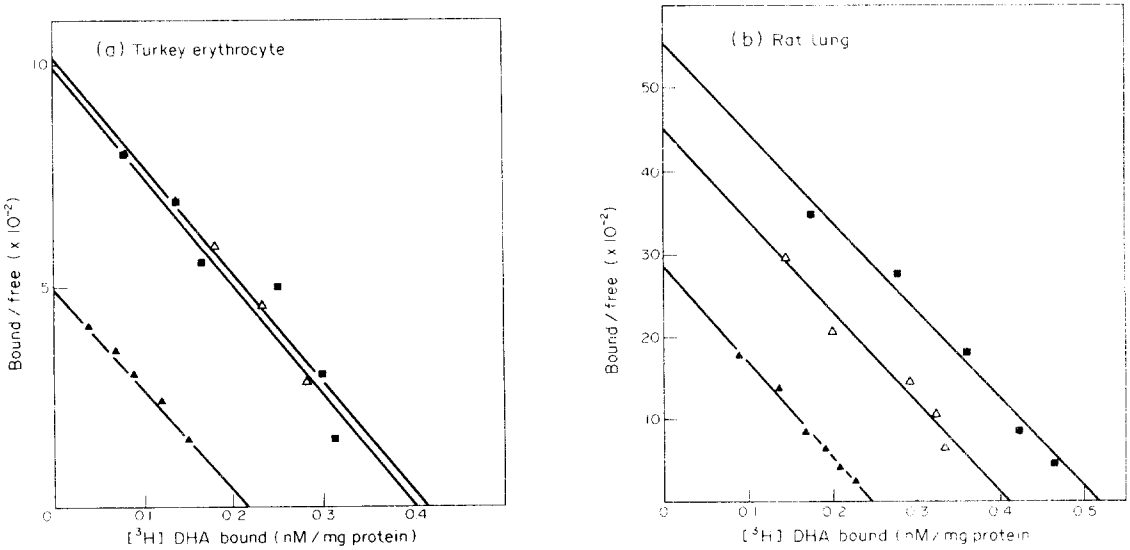

Fig. 1. Scatchard analysis [21] of the specific binding of [^{3}H]-DHA. (a) Turkey erythrocyte membranes: (■—■) control (B_{max} = 418 fM/mg protein, K_d = 4.2 nM), (▲—▲) after isoproterenol/NEM treatment (B_{max} = 223 fM/mg protein, K_d = 4.3 nM) (△—△) after reactivation, 30 min at 37° in buffer pH 8.6 (B_{max} = 410 fM/mg protein, K_d = 4 nM). (b) Rat lung membranes: (■—■) control (B_{max} = 519 fM/mg protein, K_d = 0.95 nM), (▲—▲) after isoproterenol/NEM treatment (B_{max} = 250 fM/mg protein, K_d = 0.87 nM), (△—△) after reactivation, 30 min at 37° in buffer pH 7.6 (B_{max} = 410, K_d = 0.92 nM). The binding assay was carried out as described under "Materials and Methods" using concentrations of [^{3}H]-DHA ranging from 1 to 20 nM. The membranes were sequentially submitted to the different treatments.

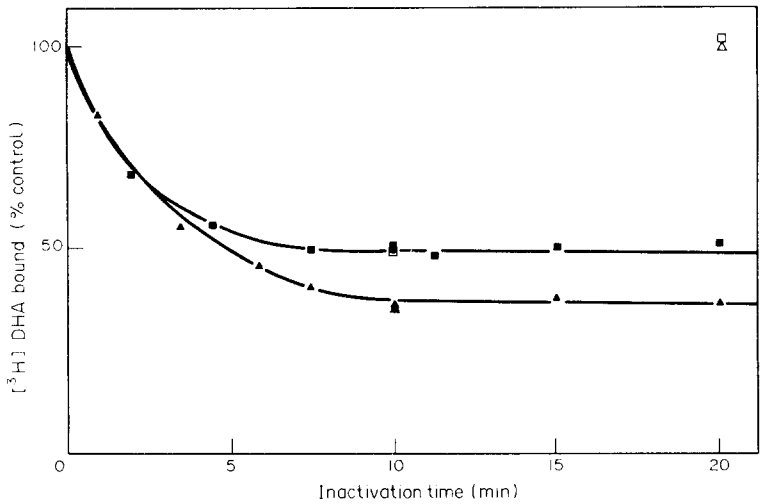

Fig. 2. Decrease in the apparent [^{3}H]-DHA specific binding to turkey erythrocyte and rat lung membrane β receptors by isoproterenol/NEM in function of the time of preincubation. The turkey erythrocyte (▲—▲) and rat lung (■—■) membranes were pretreated as described under "Materials and Methods". Control binding was not affected by NEM pretreatment for 20 min (△, □), and two successive incubations (10 min) with fresh agonist and reagent (△, □) do not increase significantly the amount receptor inactivated respectively in turkey erythrocyte and rat lung membranes.

remaining receptor sites is unaffected. As depicted by the kinetic study in Fig. 2, the above described experimental condition provides a maximal decline in receptor number for both membranes. This figure shows also that there is no effect of NEM alone, and that a second incubation with fresh agonist and reagent does not cause further decrease in receptor number. Accordingly, the ability of agonist/NEM to inactivate only part of the receptors is not due to loss in reactivity.

In rat lung membranes, there is a good correlation between the percentage of agonist/NEM sensitive sites (50%) and of high agonist affinity sites in the presence of Mg^{2+} (48%). This latter value has been calculated by computerised iteration of isoproterenol/[^{3}H]-DHA competition binding curves according to the method of Minneman *et al.* [20] (Fig. 3a). As expected, the agonist/NEM treatment actually causes the disappearance of the receptor fraction with high agonist affinity. The isoproterenol competition curve becomes uniphasic after agonist/NEM treatment (Fig. 3b) and the IC_{50}-value (1 μM) is in the same range as for the receptor population with low agonist affinity in the untreated membranes (IC_{50} = 1.2 μM). The correlation between the effect of agonist/NEM and of Mg^{2+} is difficult to establish for turkey erythrocyte membranes due to the very small difference in agonist affinity between the two receptor subpopulations [22].

For both rat lung and turkey erythrocyte membranes, the agonist/NEM-mediated decrease in receptor number can be reversed by extended incubation in fresh buffer. As appears from Table 1, this reactivation process becomes more efficient upon increasing the pH and the temperature, and upon addition of high concentrations of guanine nucleotides such as GTP. Incubation of the agonist/NEM pretreated membranes at 37°, in slightly alkaline medium (pH 8.6) for 30 min, restores the number of receptor binding sites to 88% (turkey erythrocyte) and 93% (rat lung) of their initial value (Table 1). The reactivated receptors exhibit the same affinity for [^{3}H]-DHA as the native and the agonist/NEM resistant receptors (Fig. 1).

The presence of disulfide bonds at the ligand binding site of turkey erythrocyte β_1-adrenergic receptors has already been demonstrated by the ability of the reducing agent dithiothreitol (DTT) to inactivate these receptors and of agonists and antagonists to protect them from DTT-inactivation [17]. As shown in Fig. 4, DTT (1 mM, 10 min, 30°) causes almost complete inactivation of the turkey erythrocyte β_1-receptors as well as of the rat lung β_2-receptors. For both membranes, the receptors are equally sensitive to DTT in native membranes, following agonist/NEM pretreatment and following subsequent receptor reactivation (Figs. 4a and b). This implies that both the agonist/NEM sensitive and resistant receptor populations contain essential disulfide bonds. Moreover, the disulfide bonds of both receptor populations have not been affected by the agonist/NEM pretreatment.

The reactivation treatments have as side effect that they partly impair the extent of receptor–Ns coupling in both rat lung and turkey erythrocyte membranes (Table 2). To answer the question whether the reactivated receptors are still able to undergo Ns-coupling, we have searched for experimental conditions which allow a satisfactory compromise between the optimal percentage of receptor reactivation and the coupling loss.

For turkey erythrocytes such a compromise can be reached by treating the membranes at 37° at pH 8.6. Indeed, [^{3}H]DHA binding is reduced to 43% of control after isoproterenol/NEM pretreatment and enhanced to 88% of control following reactivation under the described conditions. These reactivation conditions thus allow the reappearance of 79% of the lost sites (Table 1), while there is a 36% decline in the coupling capability in control membranes

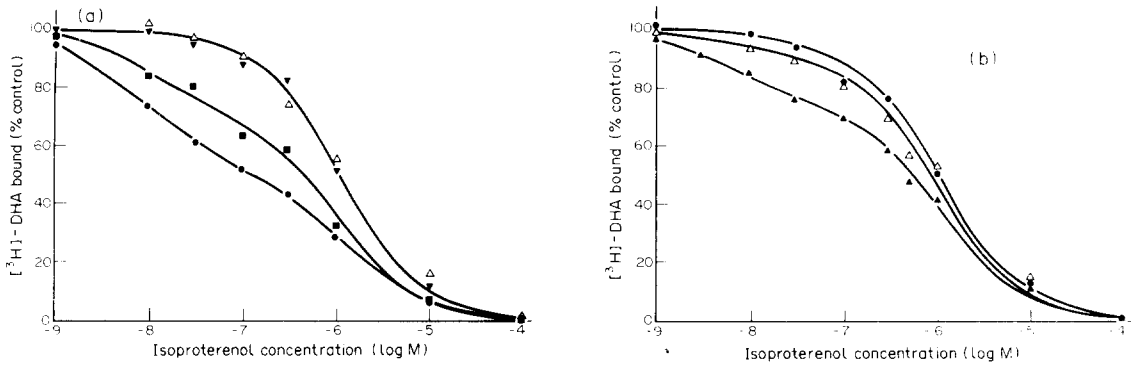

Fig. 3. Isoproterenol/[^{3}H]-DHA competition curves on rat lung membranes. Rat lung membranes were incubated with 5nM [^{3}H]-DHA and increasing concentrations of isoproterenol for 10 min. Control (100%) binding was measured in the presence of buffer only. The Hofstee plots of the competition data were analyzed by computerized iteration [20]. (a) Control membranes: (●—●) no preincubation: 48% high affinity binding sites (IC_{50} = 8.5 nM) and 52% low affinity sites (IC_{50} = 1.2 μM); (△—△) no preincubation but competition done in the presence of GTP (1 mM): one type of sites (IC_{50} = 1.2 μM); (▼—▼) membranes after 30 min incubation at 37° in buffer pH 8.6: one type of site (IC_{50} = 1.2 μM); (■—■) membranes after 30 min incubation at 30° in buffer pH 7.6 containing 0.5 mM GTP: 30% high affinity binding sites (IC_{50} = 11 nM) and 70% low affinity binding sites (IC_{50} = 1.1 μM). (b) Isoproterenol/NEM pretreated membranes: following the pretreatment, there is a decrease of 52% of the binding sites for [^{3}H]-DHA. The membranes are then submitted to a reactivation of 30 min at 30° in buffer pH 7.6 containing 0.5 mM GTP (reactivation of 83%). (●—●) No reactivation treatment: one type of site (IC_{50} = 1 μM); (▲—▲) competition in the absence of GTP: 26% high affinity sites (IC_{50} = 8 nM) and 74% low affinity sites (IC_{50} = 1.1 μM); (△—△) in the presence of 1 mM GTP: one type of site (IC_{50} = 1.15 μM). Values are the means of three independent experiments. The membranes are washed twice before undergoing the competition binding.

(Table 2). Under these conditions, it can be demonstrated that the reactivated receptors are again able to undergo Ns-coupling. When the total amount of receptors in control membranes is taken as a 100% basis, 26% of them are again agonist/NEM-sensitive in the reactivated preparation (Fig. 5). This value is close to the theoretical 29% corresponding to the situation wherein all the reactivated receptors are coupling prone (i.e. percent of reactivated receptors (45%, Table 1) × fraction of receptors whose coupling capability has not been altered by the reactivation treatment (0.64)).

In contrast, in rat lung membranes, the receptor-Ns coupling appears to be much more sensitive to the reactivation treatment. In the conditions optimal for turkey erythrocytes (pH 8.6, 37°, 30 min) the loss in coupling capability was almost total. This is evidenced by the loss of the high affinity sites in agonist binding (Fig. 3a) and of the agonist/NEM sensitivity (only 14% of the receptors were still

Table 1. [^{3}H]-DHA binding to turkey erythrocyte and rat lung membranes after preincubation with isoproterenol/NEM and different reactivation treatments

Incubation of membranes after isoproterenol/NEM pretreatment	[^{3}H]-DHA bound (% of control)	
	Turkey erythrocyte	Rat lung
No incubation	43 ± 8	50 ± 5
Reactivation (30 min)		
30°, pH 7.6	55 ± 8	58 ± 9
30°, pH 7.6 + GTP (0.5 mM)	79 ± 5	88 ± 4
37°, pH 7.6	77 ± 4	86 ± 5
37°, pH 7.6 + GTP (0.5 mM)	82 ± 10	89 ± 3
37°, pH 8.6	88 ± 6	98 ± 3

After incubation with isoproterenol/NEM or with buffer alone (for control membranes), the membranes are washed thrice and resuspended in the reactivation buffers. An aliquot of treated and of control membranes is removed to determine the residual binding of [^{3}H]-DHA. Reactivation occurred for 30 min at the indicated temperatures and pH. The percentage bound [^{3}H]-DHA was calculated for each sample with respect to control membranes submitted to the same reactivation treatment. The total receptor number, in the control membranes submitted to the same reactivation treatment, is not significantly affected (data not shown).

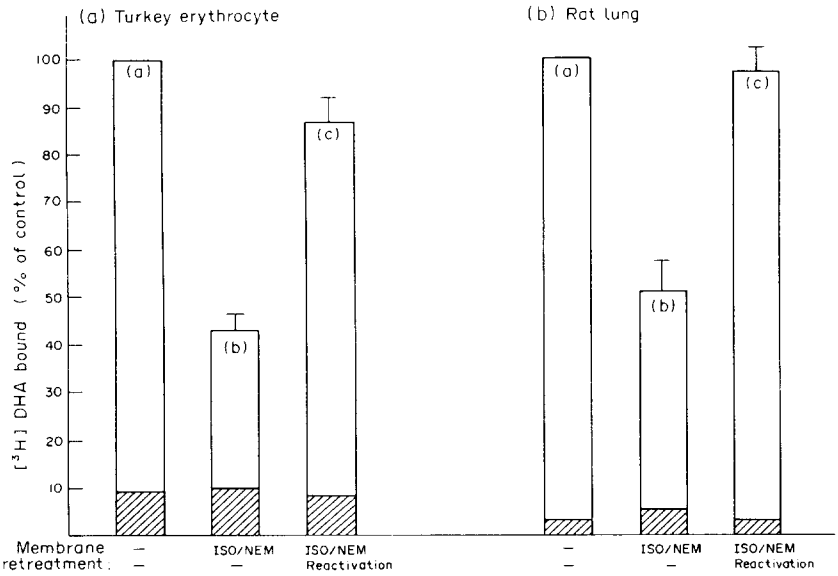

Fig. 4. Effect of the treatment with isoproterenol/NEM and reactivation upon the β-adrenergic receptor number. (a) Turkey erythrocyte membranes and (b) rat lung membranes: column a, control membranes; column b, the membranes were treated at 30° with isoproterenol/NEM and washed; column c, following the agonist/NEM treatment and washing, aliquots of the membranes were further incubated for 30 min at 37° in Tris buffer pH 8.6. All incubation mixtures were assayed for [³H]-DHA binding at concentrations of 10 nM for turkey erythrocyte and 5 nM for rat lung membranes. The hatched areas represent the remaining specific binding after treatment of the membranes with DTT (1 mM, 10 min).

isoproterenol/NEM sensitive, Table 2). The compromise here is found to be: 30 min incubation at 30° in buffer at pH 7.6 containing 0.5 mM GTP. As 50% of [³H]DHA binding sites are recovered following isoproterenol/NEM pretreatment and 88% following reactivation treatment, these conditions allow thus the reappearance of 76% of the lost sites (Table 1 and Fig. 3b), along with a 22% to 38% reduction in the coupling capability. The two latter values were respectively obtained by the agonist/NEM-sensitivity method (Table 2) and by analysis of complex isoproterenol/[³H]DHA competition binding curves (Fig. 3b).

As for the turkey erythrocyte receptors, the majority of the reactivated rat lung receptors are still coupling-prone. Whereas β-adrenergic agonists only bind with low affinity to the agonist/NEM treated rat lung membranes, the subsequent reactivation treatment results again in complex agonist binding curves with high and low affinity components in the presence of Mg^{2+}. With [³H]-DHA binding to control membranes as a 100% basis, 24% of them show again high agonist affinity in the reactivated preparation (Fig. 3b). This value is close to the 26% which can be expected in case of complete recovery of the coupling capability (i.e. percent of reactivated receptors (43%, Fig. 3b) × fraction of receptors with intact coupling capability after the reactivation treatment (0.61, Fig. 3a)).

Our data thus suggest that the great majority of the restored receptors are again able to undergo functional coupling to Ns in both the turkey erythrocyte and the rat lung membranes.

DISCUSSION

β-Adrenergic agonists mediate the functional coupling between their receptors and Ns. This coup-

Table 2. Sensitivity of the R–Ns coupling towards the reactivation treatments

Treatment of membranes (30 min)	Turkey erythrocyte		Rat lung	
	Iso/NEM sensitive sites (% of control)	% Loss coupling	Iso/NEM sensitive sites (% of control)	% Loss coupling
None	56 ± 8	0	50 ± 5	0
30°, pH 7.6 + GTP (0.5 mM)	48 ± 6	14	39 ± 5	22
37°, pH 7.6	42 ± 6	25	26 ± 4	48
37°, pH 8.6	36 ± 4	36	14 ± 4	73

Turkey erythrocyte and rat lung membranes are submitted to the reactivation treatments (as indicated), and, after the centrifugation and washing steps, the membranes are incubated with isoproterenol/NEM or with buffer alone (for control membranes). The percentage bound [³H]-DHA was calculated for each sample with respect to control membranes.

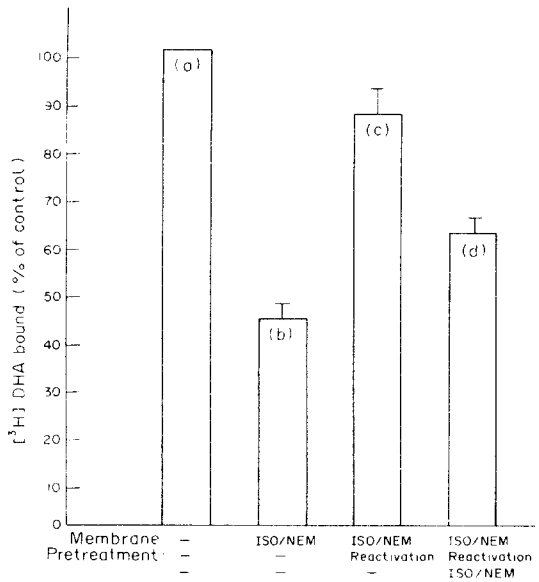

Fig. 5. Effect of isoproterenol/NEM on turkey erythrocyte membranes after inactivation and reactivation pretreatments: column a, control membranes (untreated); column b, isoproterenol/NEM treated membranes (as described); column c, isoproterenol/NEM treated membranes followed by reactivation (30 min at 37° in buffer pH 8.6), column d, isoproterenol/NEM inactivation of the reactivated membranes.

ling goes along with the exposure of sulphydryl groups which can be alkylated by NEM to give rise to a receptor state which can no longer be recognized by radioligands. Whereas all the published data agree with the above model, it is still a matter of speculation whether these sulphydryl groups become exposed at Ns or at the surface of the receptor itself. The different published interpretations, summarized below, also disagree about the molecular mechanism by which NEM alkylation is able to hamper radioligand binding to the receptor.

It was first suggested that, in turkey erythrocyte membranes, the β-receptor possesses a hidden sulphydryl group which becomes exposed as a result of the formation of the H.R.Ns complex, and which is then susceptible to alkylation by NEM [14]. The inactivation itself would be due to alkylation of this sulphydryl group, essential for radioligand binding, at the surface of the receptors (model 1).

Heidenreich *et al.* [11] adopted this hypothesis in part, but suggested moreover a scheme of action according to which the formation of the H.R.Ns complex, in rat lung membranes, results in the reduction of a disulfide bond present in the receptor binding site. The alkylation of the freed —SH groups by NEM will prevent their reoxidation to an S—S bond and hence maintain the agonist bound to the receptor, thus preventing the subsequent radioligand assay of the receptor (model 2).

The formation of a slow-dissociating agonist–receptor complex in the presence of NEM, on β_1-receptors of turkey erythrocyte membranes, was confirmed by Korner *et al.* [15]. These authors were, however, able to demonstrate that the number of β-receptor binding sites can regain its original value after incubation of agonist/NEM pretreated membranes in alkaline buffers. Based on this process of "receptor reactivation" and other data, the authors proposed that NEM traps the H.R.Ns complex in a locked conformation by alkylating —SH group(s) located on Ns and not on the receptor itself (model 3).

Here, we show that receptor reactivation can be achieved for both turkey erythrocyte β_1-receptors and rat lung β_2-receptors. Since alkylation by NEM is a covalent process [23], and hence irreversible, the possibility of reactivation implies that NEM does not inactivate the β-adrenergic receptors via steric hindrance at the ligand binding site (model 1) but rather that it freezes the receptor in a slow agonist-dissociating conformation.

Heidenreich's model, wherein NEM alkylates a sulphydryl group emerging from the reduced disulfide bond of the receptor (model 2), does not necessarily exclude the dissociation of bound agonist at high pH. However, since radioligand binding requires an intact disulfide bond at the receptor binding site, the model 2 can hardly explain the process of receptor reactivation. Moreover, we show here that, for both turkey erythrocyte and rat lung membranes, the reactivated receptors are dithiothreitol (DTT) sensitive in the absence of β-adrenergic ligands and thus still contain an intact disulfide bond at their binding site (Fig. 4).

There is thus strong evidence against the ability of NEM to alkylate a sulphydryl group resulting from a reduced disulfide bond at the ligand binding site of the receptor. This does not, however, impair the hypothesis that β-adrenergic agonists act as reducing agents and open this disulfide bond. Interestingly enough, the standard reduction potential for diphenol (present in potent β-adrenergic agonists)/o-quinone is 0.35 V [24]. This value is similar to the standard reduction potential for DTT (red/ox = 0.33 V) [25]. It cannot therefore be excluded that β-adrenergic agonists might promote the reductive cleavage of the receptor disulfide bond, and that this event is implicated in the process of receptor activation.

Since the reactivated receptors show the original radioligand and agonist binding characteristics, the same reactivity towards the reagent DTT, and are still able to undergo functional coupling, it is likely that NEM alkylates the sulphydryl groups which become exposed at the surface of Ns rather than the receptor itself. This interpretation is also in complete agreement with our previous data establishing the necessity of H–R–Ns complex formation for the agonist/NEM effect. As a striking example, digitonin-solubilized turkey erythrocyte β-adrenergic receptors are no longer agonist/NEM sensitive [14].

That coupling still occurs in the reactivated β-adrenergic system sheds some light on the question of the functional heterogeneity of the receptors. A plausible explanation is that of a stoichiometrical limitation of Ns. However, although the functional stoichiometry of the receptors, of the Ns proteins and of the adenylate cyclase component is still obscure, the ability of the reactivated receptors to undergo coupling to Ns suggests that the Ns components might be present in excess with respect to

the receptors. Alternative causes for the limitation in the agonist/NEM effect could be that the resistant receptors cannot interact with Ns in a productive manner due to differences either in the receptor structure or in its membrane microenvironment. In the context of the second assumption, it has already been demonstrated that factors which perturb the membrane, such as the incorporation of fillipin [26], hamper the Mg^{2+}-dependent formation of high agonist affinity sites, and hence receptor–Ns coupling. Furthermore, all the published studies report some variability in the percentage of coupling-prone receptors from one membrane preparation to another. We are therefore tempted to speculate that differences in receptor microenvironment might play a key role in determining whether or not the receptors will have access to Ns.

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