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Inactivation of β-Adrenergic Receptors by *N*-Ethylmaleimide: Permissive Role of β-Adrenergic Agents in Relation to Adenylate Cyclase Activation

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SUMMARY

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The β_1-adrenergic receptors of turkey erythrocyte membranes have been identified by the specific binding of the radiolabeled antagonist (−)-[^{3}H]dihydroalprenolol. Binding of β-adrenergic agonists to these receptor sites sensitizes them to inactivation by the alkylating agent *N*-ethylmaleimide. A dose- and time-dependent decrease of 45 to 60% of the sites is commonly observed. Binding of (−)-^{3}H-dihydroalprenolol and β-adrenergic agonists to the remaining sites occurs with the same characteristics as for the untreated receptor population. Kinetic experiments reveal that the rate of inactivation is proportional to the concentration of *N*-ethylmaleimide (between 5.5 and 450 μM). In contrast, the rate of inactivation reaches a plateau value when increasing the concentration of the agonist. The rate of inactivation is half-maximal in presence of 1.3 μM (−)-epinephrine or 20 μM (+)-epinephrine. This marked stereospecificity, along with the close identity of the above concentrations with the equilibrium dissociation constant (K_D) of the epinephrine isomers for binding to the β-receptor (i.e., 2.0 μM for (−)-epinephrine and 21 μM for (+)-epinephrine) indicate that *N*-ethylmaleimide inactivates the agonist-bound form of the receptor. The second-order rate constant (k_2) of the inactivation process, in the presence of 15 β-adrenergic ligands, was found to correlate with their capability to stimulate the adenylate cyclase activity, i.e., "intrinsic activity." Since all tested ligands were able to cause a complete and dose-dependent displacement of bound (−)-[^{3}H]dihydroalprenolol, it is likely that both the intrinsic activity and k_2 of each adrenergic ligand reflect an inherent property of the ligand-bound receptor. The proportionality between k_2 and the intrinsic activity further suggests that β-adrenergic agonists "induce" or "favor" a conformational change of the receptor, resulting in adenylate cyclase activation and the uncovering of an alkylable group which becomes exposed to *N*-ethylmaleimide in the active conformation.

INTRODUCTION

Catecholamine hormones such as epinephrine and norepinephrine activate the adenylate cyclase enzyme by binding to β-adrenergic receptors located at the cell membrane (1). These receptors have been subdivided in the β_1 and β_2 subclasses on the basis of differences in affinity for norepinephrine and epinephrine and by obtaining selective β_1 (e.g., practolol) and β_2 (e.g., butoxamine) blocking drugs (2, 3). Detailed structure–activity relationships have been determined for β-adrenergic drugs to stimulate the adenylate cyclase enzyme by binding to either β_1- or β_2-adrenergic receptors (4–7). Such studies have resulted in the classification of these drugs as full agonists, partial agonists (causing partial cyclase activation) and antagonists (causing no cyclase activation themselves, but inhibiting the agonist-induced activity).

In the past few years, several radiolabeled antagonists such as (−)-[^{3}H]-dihydroalprenolol, [^{3}H]propranolol and [^{125}I]iodohydroxybenzylpindolol have been synthesized and successfully applied for the labeling of β-adrenergic

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receptors in different tissues (8). In previous reports, we have shown that the β_1-adrenergic receptors on turkey erythrocyte membranes are labeled with high specificity by binding of (−)-[^{3}H]-dihydroalprenolol (9, 10). Binding occurs to a single class of noncooperative sites (0.2 to 0.3 pmole/mg protein) with an equilibrium dissociation constant (K_D) of 8 of 12 nM and with fast association and dissociation kinetics ($t_{1/2}$ less than 30 sec). The availability of these labels has permitted the successful solubilization of the β-adrenergic receptors from several tissues (9, 11, 12) and the purification of the β_1-receptors from turkey erythrocytes by affinity chromatography (9). The β-receptors were shown to be discrete membrane components, distinct from the cyclase enzyme (9, 11, 12).

The chemical characterization of the receptors has been initiated by use of group-specific reagents. In this respect, the most striking result is that the β_1-receptors from turkey erythrocytes and C6 glioma cells contain essential disulfide bonds (10, 13), apparently absent from the β_2-receptor of frog erythrocytes (14). A distinction between agonist and antagonist binding was outlined recently by the observation that agonists but not antagonists cause "desensitization" of the β-receptors in many tissues (14–16), probably by inducing a "high affinity" state of the receptor (17).

In a recent communication (18), we have shown that the alkylating agent *N*-ethylmaleimide causes a substantial decrease in the amount of β_1-adrenergic receptor sites in turkey erythrocyte membranes in the presence of agonists, but not of antagonists. Either agonists or *N*-ethylmaleimide alone do not affect the amount of receptor sites. We now describe detailed studies intended to evaluate the respective role of the agonists and the agent *N*-ethylmaleimide in this inactivation process, as well as the relationship between this process and agonist-induced adenylate cyclase activation. These studies reveal that the cyclase activation and the sensitivity of the receptors to *N*-ethylmaleimide are closely linked phenomena, likely due to a conformational change of the receptor.

MATERIALS AND METHODS

Materials The following were obtained as generous gifts: (+)- and (−)-epinephrine bitartrate, (−)-isoproterenol bitartrate, β-deoxyisoproterenol and (−)-nordefrin (Sterling Winthrop); (+)- and (−)-propranolol (Imperial Chemical Industries, U.K.); (±)-alprenolol hydrochloride (Ciba-Geigy); butoxamine hydrochloride (Burroughs Wellcome); orciprenaline (Badrial); fenoterol (Boehringer-Ingelheim); trimethoquilol (Tanabe Seiyaku Co., Japan); (−)-phenylephrine hydrochloride, (−)-norepinephrine bitartrate, dopamine hydrochloride, ATP and cysteine were purchased from Sigma Chemical Company, *N*-ethylmaleimide was from Fluka A. G., creatine phosphokinase from Boehringer, cyclic AMP and phosphocreatine from Calbiochem. [α-^{32}P]ATP (6–29 Ci/mmole) and (−)-[^{3}H]-dihydroalprenolol (33 Ci/mmole) were obtained from New England Nuclear Corporation and cyclic [8-^{3}H]AMP (13 Ci/mmole) from the Commissariat à l'Energie Atomique (Saclay, France).

All other chemicals were of analytical grade.

Preparation of turkey erythrocyte membranes: Turkey erythrocyte membranes were prepared according to Øye and Sutherland (19) with small modifications as described elsewhere (10). Membranes were suspended in 10 mM Tris–HCl (pH 7.4)/145 mM NaCl/2 mM $MgCl_2$ containing 10% (v/v) glycerol, and stored in liquid nitrogen until use. Protein determinations were performed by the method of Lowry et al. (20) using bovine serum albumin as the standard.

Adenylate cyclase assay Adenylate cyclase activity was measured as described (9). The assay medium contained 1 mM [α-^{32}P]ATP ($1.5.10^6$ cpm), 7 mM $MgCl_2$, 1 mM EDTA, 1 mM cyclic AMP, 50 mM Tris–HCl (pH 7.6), an ATP-regenerating system (consisting of 25 mM phosphocreatine and creatine phosphokinase 1 mg/ml), β-adrenergic agents at the indicated concentration, and 60 μg of membrane protein in a final volume of 60 μl. Freshly prepared solutions of the β-adrenergic agents were added to the medium at 0° less than 20 min before each assay. Incubation was initiated by the addition of the enzyme and performed for 20 min at 30°. Results are the mean of triplicate determinations and are expressed in nmol cyclic AMP formed in 20 min/mg of protein.

Membrane pretreatment with β-adrenergic agents and N-ethylmaleimide (NEM):[2] Unless otherwise stated, the preincubation medium contained 75 mM Tris–HCl (pH 7.4), 25 mM $MgCl_2$, 50 μM NEM, β-adrenergic agents at the indicated concentration and 2 mg of membrane protein in a final volume of 1 ml. The preincubation was initiated by addition of a prewarmed solution of NEM (30°) to an equilibrated mixture of β-adrenergic ligand and membrane receptor (obtained by shaking a mixture of ligand and membranes for 2 min at 30°), and performed for various periods of time at 30°. The preincubation was terminated by addition of 100 μl of 20 mM cysteine. Under the given conditions, cysteine did not affect (−)-[^{3}H]-dihydroalprenolol binding to the β-adrenergic receptor sites. The mixture was then transferred into a 1.5-ml micro-test tube (Eppendorf) and centrifuged for 1 min at 1200 rpm.

After removal of the supernatant by aspiration, the preincubated membranes were resuspended into 1 ml of 75 mM Tris–HCl (pH 7.4)/25 mM $MgCl_2$ and centrifuged for 1 min. This washing step was repeated two to four times.

Binding of (−)-[^{3}H]-dihydroalprenolol ((−)-^{3}H-DHA): Following preincubation and washing, 0.4 mg of membrane protein was incubated with 10 nmole (−)-^{3}H-DHA for 10 min at 30° in 75 mM Tris–HCl (pH 7.4)/25 mM $MgCl_2$ in a final volume of 200 μl. At the end of the incubation, triplicate 50-μl aliquots were diluted at 0° in 4 ml of the same buffer and filtered under reduced pressure through glass fiber filter disks (Whatman GF/F, 2.5 cm in diameter). Filters were washed rapidly with 6 ml of ice-cold buffer, placed in 20-ml polyethylene scintillation vials with 1 ml of 1 N HCl and 10 ml Aqualuma scintillation fluid (Lumac) and counted for 10 min in a Packard liquid scintillation spectrometer; 1 pmole (−)-^{3}H-DHA bound/mg protein corresponds to 3400

[2] The abbreviations used are: NEM, *N*-ethylmaleimide; (−)-^{3}H-DHA, (−)-[^{3}H]dihydroalprenolol.

cpm. Dilution and washing did not noticeably affect (−)-^{3}H-DHA binding under the present conditions. The half-life of the (−)-^{3}H-DHA and β-adrenergic receptor complex equals 25 min at 0° and the extent of bound (−)-^{3}H-DHA does not vary when filters are washed with 4 to 20 ml of ice-cold buffer. In all experiments, the amount of nonspecific (−)-^{3}H-DHA binding was determined by incubating membranes and (−)-^{3}H-DHA in the presence of 1 μM (±)-alprenolol. Specific binding was obtained by subtracting nonspecific binding from total binding, and approximated 85 to 90% of the total binding. In all figures and tables, bound (−)-^{3}H-DHA refers to specific binding as defined above.

(−)-^{3}H-DHA saturation binding data were obtained by incubating the membranes with 1 to 100 nM tracer. Analysis of these data according to the method of Scatchard (21) allowed calculation of the total amount of binding sites (B_{max}) and the equilibrium dissociation constant for binding (K_D).

RESULTS

Specific (−)-^{3}H-DHA binding to turkey erythrocyte membranes occurs to a single class of noncooperative sites, previously identified as β_1-adrenergic receptors (9, 10). Pretreatment of these membranes with a combination of NEM and β-adrenergic agonists causes a net reduction in the number of binding sites (Table 1). Maximal inactivation can be achieved by pretreatment with 1 mM NEM plus 0.1 μM (−)-isoproterenol at 30° for 3 min or longer (18). A 10-min pretreatment was chosen for the calculation of the total amount of inactivable binding sites. This amount ranges between 45% (Table 1) and 60%, depending on the membrane preparation, and is not affected by prolonged storage on the membranes in liquid nitrogen up to 2 months.

The β-adrenergic agonists still cause a complete and dose-dependent displacement of (−)-^{3}H-DHA binding to the remaining receptors. The equilibrium dissociation constants (K_D) for (−)-^{3}H-DHA and the β-adrenergic agonists for binding to the remaining sites are nearly the same as for binding to the untreated receptor population (Table 1). Accordingly, the typical order of affinities for agonist binding to β_1-adrenergic receptors (i.e., isoproterenol > norepinephrine ≥ epinephrine) as well as the stereospecificity are preserved.

TABLE 1

Characteristics of (−)-^{3}H-DHA binding sites in native and NEM plus (−)-isoproterenol-treated turkey erythrocyte membranes

Membranes were either native or treated with 0.1 μM (−)-isoproterenol plus 1 mM NEM for 10 min at 30°. The total amount of (−)-^{3}H-DHA binding sites (B_{max}) and the equilibrium dissociation constant for binding (K_D) were calculated as described under Materials and Methods. Agonist $K_{D(I)}$ values were calculated from the concentration that causes half-maximal decrease of (−)-^{3}H-DHA binding, according to Cheng and Prusoff (22). Values are the means ± SE of two to three experiments.

Binding characteristics	Turkey erythrocyte membranes	
	Untreated	Treated with NEM + (−)-isoproterenol
(−)-^{3}H-DHA binding		
B_{max} (pmole/mg protein)	0.22 ± 0.1	0.12 ± 0.1
K_D (nM)	9.2 ± 0.8	8.4 ± 0.6
Displacement by agonist ($K_{D(I)}$ in μM)		
(+)-Epinephrine	21 ± 6	16 ± 2
(−)-Epinephrine	2.0 ± 0.7	1.3 ± 0.2
(−)-Norepinephrine	0.63 ± 0.08	0.50 ± 0.06
(−)-Isoproterenol	0.070 ± 0.04	0.070 ± 0.010

Time and concentration dependence of epinephrine-NEM-induced decrease in the number of (−)-^{3}H-DHA binding sites. When turkey erythrocyte membranes are exposed to (−)-epinephrine plus NEM at 30°, the number of (−)-^{3}H-DHA binding sites decreases timewise, until a plateau is reached which, for the investigated membrane preparation, corresponds to 50% of the initial sites (Figs. 1, 2). When the amount of sensitive sites is plotted on a logarithmic scale (Figs. 1, 2: inset), the decrease appears to be linearly related to the preincubation time. Therefore, the inactivation proceeds according to apparent first-order kinetics. The apparent first-order rate constant (k_{ob}) is defined by the equation

$$\ln B/B_0 = -k_{ob} \cdot t, \qquad [1]$$

which is represented by the inset of Figs. 1 and 2. B and B_0 represent the amount of inactivable binding sites (i.e., 100 × (observed binding − resistant binding)/total binding) at time t and $t = 0$, respectively.

A close examination of the dependence of k_{ob} on the epinephrine and NEM concentration provides information about the role of both compounds in the inactivation process. In the experiment shown in Fig. 1, membranes are treated with increasing concentrations of NEM (5.5

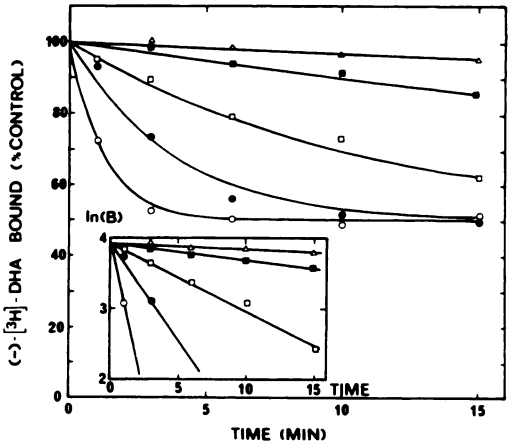

FIG. 1. *Inactivation of (−)-^{3}H-DHA binding sites by (−)-epinephrine plus increasing concentrations of NEM*

Membranes were pretreated with 2 μM (−)-epinephrine in addition to NEM (5.5 μM (△——△), 17 μM (■——■), 50 μM (□——□), 150 μM (●——●), 450 μM (○——○)) for the indicated periods of time at 30°, washed three times and incubated with 10 nM (−)-^{3}H-DHA. Control binding refers to binding of (−)-^{3}H-DHA to membranes that have been pretreated with (−)-epinephrine plus NEM for less than 10 sec (i.e., 0.135 pmole/mg protein). Binding to membranes pretreated for 15 min with buffer only, with 2 μM (−)-epinephrine, or with 450 μM NEM agrees with control binding within 5%. *Inset*: Semilogarithmic representation of the same binding data. B represents the amount of inactivable binding sites i.e., 100 × (observed binding-resistant binding)/total binding, at time t. Resistant binding is 50% of the total for the considered membrane preparation.

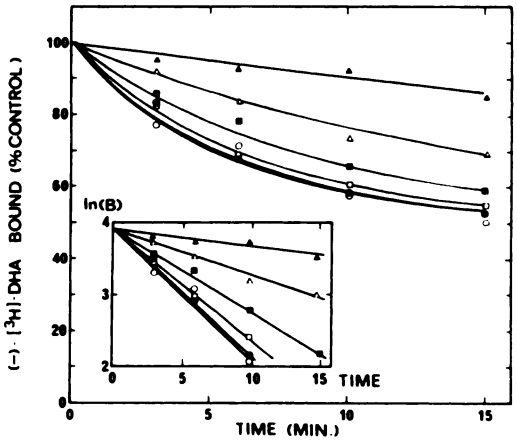

FIG. 2. *Inactivation of (−)-^{3}H-DHA binding sites by NEM plus increasing concentrations of (−)-epinephrine*

Membranes were pretreated with 50 μM NEM in addition to (−)-epinephrine 0.22 μM (▲——▲), 0.67 μM (△——△), 2 μM (■——■), 6 μM (□——□), 18 μM (●——●), 54 μM (○——○)) for the indicated periods of time at 30°, washed five times and incubated with 10 nM (−)-^{3}H-DHA. Control binding is defined as for Fig. 1. Pretreatment of the membranes with 54 μM (−)-epinephrine for 15 min did not cause more than 5% inhibition of the binding activity. *Inset*: Semilogarithmic representation of the same binding data as stated for Fig. 1.

to 450 μM) in addition to a steady amount of (−)-epinephrine (2 μM) for different periods of time. Whereas the total amount of inactivable (−)-^{3}H-DHA binding sites remains unchanged (i.e., 50%), there is a net increase of the inactivation rate when the NEM concentration is raised. As depicted in Fig. 3A, there is a linear dependence of log (k_{ob}), calculated according to the inset of Fig. 1, on log (NEM concentration). The slope equals 1.04 (r = 0.92), indicating that k_{ob} is directly proportional to the NEM concentration (i.e., $k_{ob} = 2.10^3 \times$ [NEM]), and accordingly, that the inactivation process is monomolecular with respect to NEM.

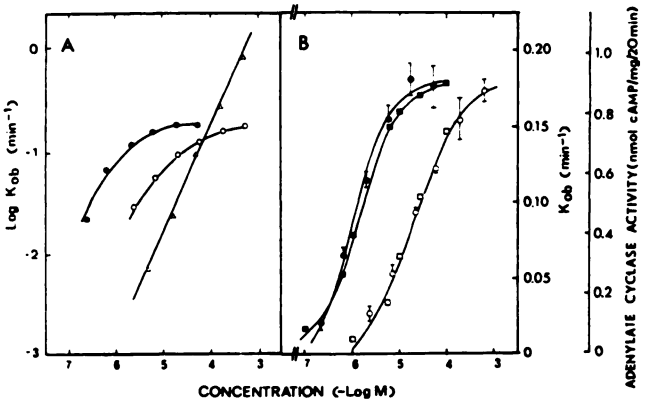

FIG. 3. *Rate of inactivation of (−)-^{3}H-DHA binding sites in function of the epinephrine- and NEM concentration*

k_{ob} represents the apparent first-order rate constant of the inactivation reaction, and is calculated according to equation [1]. (A) k_{ob} as a function of increasing concentrations of NEM (△——△, from data shown in Fig. 1). The (−)-epinephrine concentration was constant (i.e., 2 μM). k_{ob} as a function of increasing concentrations of (−)-epinephrine (●——●, from data presented in Fig. 2) and (+)-epinephrine (○——○, from analogous, unpublished observations). The NEM concentration was constant in both cases (i.e., 50 μM). Both scales are logarithmic. (B) k_{ob} values and adenylate cyclase activities as functions of increasing concentrations of (−)-epinephrine (●——● for k_{ob}, ■——■ for cyclase) and (+)-epinephrine (○——○ for k_{ob}, □——□ for cyclase). k_{ob} values are the same as for (A). Only the abscissa is logarithmic.

When membranes are treated with increasing concentrations of (−)-epinephrine (0.22 to 54 μM) in addition to a steady amount of NEM (50 μM), the rate of inactivation is also increased (Fig. 2). However, there is no linear dependence of log (k_{ob}), calculated according to the inset of Fig. 2, on log ((−)-epinephrine concentration) (Fig. 3A). In fact, log (k_{ob}) tends to reach a limit value corresponding to $k_{ob} = 0.18$ min^{-1}, upon increasing the (−)-epinephrine concentration.

The lack of direct proportionality between k_{ob} and the epinephrine concentration can, however, be explained by assuming that the receptors are only sensitive to NEM when bound to epinephrine. This assumption is supported by the following data.

(i) For each tested concentration of (−)-epinephrine, there is a close correspondence between the adenylate cyclase activation and k_{ob} (Fig. 3B). Maximal k_{ob} values are observed for (−)-epinephrine concentrations which induce maximal adenylate cyclase activation (i.e., for concentrations exceeding 20 μM). Half-maximal k_{ob} (0.09 min^{-1}) occurs in presence of 1.3 μM (−)-epinephrine, which is very close to the K_D values of this ligand for binding to the β-adrenergic receptor as found by either adenylate cyclase activation (K_D = 1.6 μM) or displacement of (−)-^{3}H-DHA binding (K_D = 2.0 μM, see Table 1).

(ii) The dependence of k_{ob} on the concentration of the dextrorotary isomer (+)-epinephrine has been investigated under the same conditions as for (−)-epinephrine (data not shown). The results are summarized in Fig. 3. Whereas k_{ob} versus epinephrine concentration curves of both isomers are almost superimposable, the curve corresponding to (+)-epinephrine is shifted to the right by about one order in magnitude. This difference reflects the well-known stereospecificity of adrenergic ligands for binding to the β-adrenergic receptors. Here again, half-maximal k_{ob} occurs in presence of 20 μM (+)-epinephrine (Fig. 3B), which is equal to its K_D value for the receptor found by adenylate cyclase activation (K_D = 20 μM) and binding studies (K_D = 21 μM, Table 1). According to the above observations, inactivation of the (−)-^{3}H-DHA binding sites by NEM plus epinephrine is monomolecular with respect to both NEM and the (sensitive) sites which are occupied by the ligand. The inactivation process can be described by the relation

$$v = k_2 \cdot [H \cdot R] \cdot [NEM], \qquad [2]$$

where k_2 is the second-order rate constant for the inactivation process and H·R the hormone-bound form of the inactivable receptor population.

The hormone–receptor interaction can be described as a fast reversible bimolecular equilibrium process, so that the relation between [H·R] and the concentration of hormone [H] is given by

$$[H \cdot R] = [R_t]/(1 + K_D/[H]), \qquad [3]$$

where [R_t] is the total amount of agonist plus NEM-

sensitive receptors (i.e., 45 to 60% of the total receptor population). The relation between the rate of inactivation and the concentrations of both NEM and epinephrine are given by the combination of equations [2] and [3], i.e.,

$$v = k_2 \cdot [R_t] \cdot [NEM]/(1 + K_D/[H]). \quad [4]$$

Inactivation of (−)-^{3}H-DHA binding sites by NEM plus β-adrenergic agents in function of their intrinsic activity Several β-adrenergic drugs cause only partial activation of the adenylate cyclase system (partial agonists) or no activation at all (antagonists). Figure 4 shows the adenylate cyclase activity in turkey erythrocyte membranes in the presence of increasing concentrations of the full agonist (−)-isoproterenol and some typical partial agonists such as trimethoquilol, fenoterol, terbutaline and (−)-phenylephrine. When increasing the concentration of these ligands, the adenylate cyclase activity increases until a plateau level is reached. As expected, the maximal cyclase activation is lower for the partial agonists than for (−)-isoproterenol. The maximal adenylate cyclase activation that a given ligand can induce is commonly referred to as the "intrinsic activity" (see Discussion). The intrinsic activity of (−)-isoproterenol was defined as 1.0. The intrinsic activities of 15 β-adrenergic ligands have been calculated from dose–response relationships, similar to the ones shown in Fig. 4, and are presented in Table 2, along with the concentrations which cause half-maximal adenylate cyclase activation

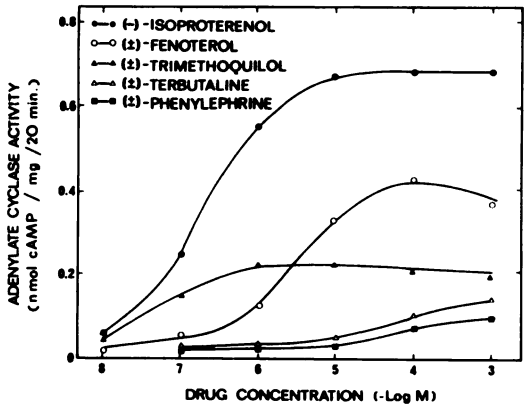

FIG. 4. *Adenylate cyclase activation by β-adrenergic agents*
Adenylate cyclase activities were measured in the presence of increasing concentrations of the full agonist (−)-isoproterenol and of the partial agonists: fenoterol, trimethoquilol, terbutaline and (±)-phenylephrine. Basal adenylate cyclase activity corresponded to 0.02 nmole cyclic AMP formed/mg of membrane protein in 20 min at 30°.

($K_{D(A)}$). When turkey erythrocyte membranes are incubated with a constant amount of (−)-^{3}H-DHA (10 nM) in the presence of increasing concentrations of the drugs listed in Table 2, a dose-dependent decrease of binding activity is observed. Typical examples are given in Fig. 5. All the β-adrenergic ligands listed in Table 2 are able to produce complete inhibition of the (−)-^{3}H-DHA binding activity, independently of their intrinsic activity. Their

TABLE 2

Rate of inactivation of (−)-^{3}H-DHA binding sites by NEM in the presence of β-adrenergic agents, compared to their intrinsic activity and affinity for the receptor

The affinity and intrinsic activity of agonists and partial agonists was determined from dose-response curves for stimulation of the adenylate cyclase activity (e.g., Fig. 4). $K_{D(A)}$ is the concentration of drug that causes half-maximal adenylate cyclase activation. The intrinsic activity corresponds to maximal cyclase activation; (−)-isoproterenol being taken as the standard (i.e., intrinsic activity = 1). Antagonists did not cause significant stimulation of the enzyme, but provoked complete inhibition of the (−)-isoproterenol stimulated cyclase activity. The affinity of β-adrenergic ligands for binding to the receptor were obtained from (−)-^{3}H-DHA binding displacement curves (e.g., Fig. 5). The equilibrium dissociation constant for binding ($K_{D(I)}$) was calculated from the concentration of ligand that caused half-maximal displacement by the method of Cheng and Prusoff (22). The rate of inactivation of the (−)-^{3}H-DHA binding sites by 50 μM NEM in the presence of β-adrenergic agents was determined from kinetic experiments (e.g., Fig. 6). The concentration of each drug was equal to its $K_{D(I)}$ value for binding. The pseudo-first-order rate constant (k_{ob}) was calculated according to equation [1]; maximal inactivation (caused by 10-min preincubation with 0.1 μM (−)-isoproterenol plus 1 mM NEM) affected 60% of the binding sites in the membrane preparation used. Values are means and ranges of two experiments.

Compound	Adenylate cyclase activation		(−)-^{3}H-DHA binding	
	Intrinsic activity	$K_{D(A)}$ (μM)	$K_{D(I)}$ (μM)	K_{ob} (min^{-1})
Agonists				
(−)-Epinephrine	1.06 ± 0.11	1.6 ± 0.1	2.0 ± 0.3	0.129 ± 0.011
(−)-Isoproterenol	1.00 ± 0.05	0.15 ± 0.6	0.07 ± 0.004	0.115 ± 0.008
(−)-Norepinephrine	0.94 ± 0.11	0.50 ± 0.02	0.63 ± 0.08	0.122 ± 0.012
Partial agonists				
(−)-Nordefrin	0.71 ± 0.04	1.6 ± 0.6	0.8 ± 0.1	0.103 ± 0.013
(±)-Fenoterol	0.64 ± 0.06	3.2 ± 0.3	3.1 ± 0.2	0.091 ± 0.012
β-Deoxyisoproterenol	0.58 ± 0.03	15 ± 1	25 ± 5	0.061 ± 0.006
(±)-Orciprenaline	0.39 ± 0.01	63 ± 11	20 ± 2	0.067 ± 0.006
(±)-Trimethoquilol	0.33 ± 0.09	0.054 ± 0.027	0.050 ± 0.007	0.070 ± 0.015
dopamin	0.25 ± 0.05	150 ± 70	250 ± 27	0.017 ± 0.004
(±)-Terbutaline	0.20 ± 0.03	20 ± 4	79 ± 14	0.029 ± 0.003
(−)-Phenylephrine	0.09 ± 0.03	30 ± 7	63 ± 11	0.004 ± 0.002
Antagonists				
(±)-Butoxamine	0.0	—	93 ± 24	0.004 ± 0.006
(−)-Propranolol	0.0	—	0.005 ± 0.001	0.000 ± 0.008
(+)-Propranolol	0.0	—	0.60 ± 15	0.005 ± 0.008
(±)-Alprenolol	0.0	—	0.015 ± 0.01	0.001 ± 0.005

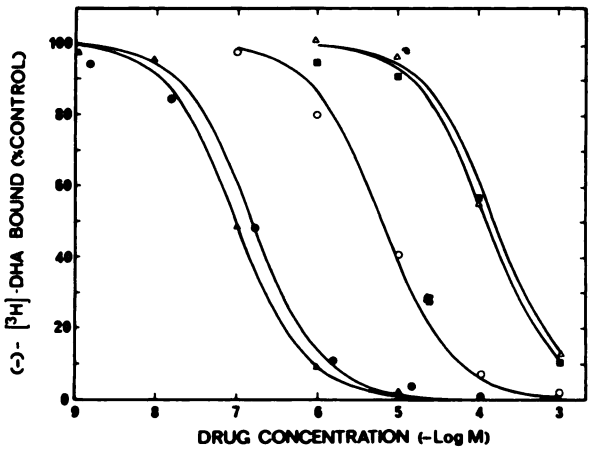

FIG. 5. *Displacement of (−)-^{3}H-DHA binding by β-adrenergic agents*

Binding of 10 nM (−)-^{3}H-DHA to turkey erythrocyte membranes was measured in the presence of increasing concentrations of the β-adrenergic drugs listed in Fig. 4. Control binding refers to (−)-^{3}H-DHA binding in the absence of drug, and was 0.15 pmole/mg protein.

equilibrium dissociation constant for binding to the receptor ($K_{D(I)}$) has been derived from their concentration which causes half-maximal inhibition according to Cheng and Prusoff (22). As shown in Table 2, $K_{D(A)}$ and $K_{D(I)}$ values are in good agreement for each compound.

Inactivation of the (−)-^{3}H-DHA binding sites by NEM has been investigated in the presence of a variety of β-adrenergic agents. For this purpose, membranes were treated for various periods of time at 30° with each ligand listed in Table 2, at a concentration equal to its $K_{D(I)}$ value for the receptor, along with a constant amount of NEM (50 μM). Despite the fact that the different ligands occupy an equal fraction of the (−)-^{3}H-DHA binding sites, a marked difference in the rate of inactivation is observed. This is well illustrated in Fig. 6. In contrast, the maximal amount of inactivable (−)-^{3}H-DHA binding sites is not affected by the choice of the ligand. Except for phenylephrine and the antagonists, the agents listed in Table 2 allow approximately 60% inhibition of the binding activity after 75 min of incubation with NEM (data not shown). The k_{ob} values have been calculated according to equation [1] for the different β-adrenergic agents and listed in Table 2.

Whereas the k_{ob} and $K_{D(I)}$ values are unrelated ($r = -0.32$), there is a linear, proportional relationship between the k_{ob} value and the intrinsic activity (Fig. 7). The correlation is highly significant ($r = 0.97$, $P < 0.001$).

Under the given experimental conditions, the factor $[R_t]\cdot[NEM]/(1 + K_D/[H])$ in the right part of Eq. [4] is equal for each investigated ligand. Accordingly, the above results indicate that the second-order rate constant for inactivation of a ligand-bound receptor by NEM, k_2, is proportional to the intrinsic activity of the considered ligand.

DISCUSSION

Simultaneous addition of the alkylator NEM and β-adrenergic agonists to turkey erythrocyte membranes causes a net decrease in the amount of (−)-^{3}H-DHA

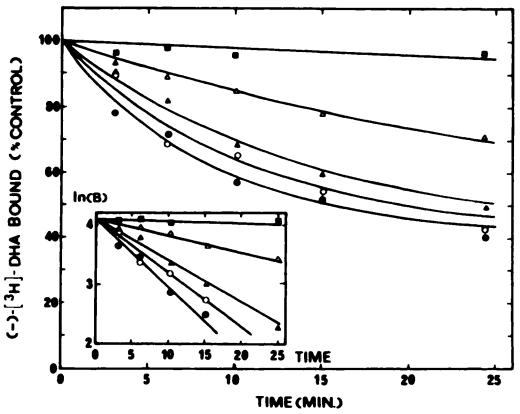

FIG. 6. *Inactivation of (−)-^{3}H-DHA binding sites by NEM in the presence of different β-adrenergic agents*

Membranes were pretreated with 50 μM NEM in addition to the β-adrenergic drugs listed in Fig. 4, for different periods of time at 30°. The concentration of each drug was equal to its equilibrium dissociation constant for binding to the receptor ($K_{D(I)}$); values are listed in Table 2. Under these conditions, each drug occupied 50% of the (−)-^{3}H-DHA binding sites. The membranes were then washed three times, and binding of 10 nM (−)-^{3}H-DHA measured. Control binding refers to binding of (−)-^{3}H-DHA to membranes which have been pretreated with NEM plus the respective β-adrenergic drug for less than 10 sec (i.e., 0.15 pmole/mg protein). Pretreatment of the membrane with the β-adrenergic agents for 15 min did not cause more than 5% inhibition of the binding activity. *Inset*: Semilogarithmic representation of the same binding data, as stated for Fig. 1. The amount of inactivable binding sites represent 60% of the total binding for the investigated membrane preparation.

binding sites, previously identified as the β_1-adrenergic receptors (9). This fall did not affect more than 45 to 60% of the binding activity, and was not reversible under our experimental conditions (18), indicating that only a well-determined subpopulation of the (−)-^{3}H-DHA binding sites can be inactivated.

The aim of this study is to give some insight concerning the respective role of the agonists and the alkylator in the inactivation process, as well as the relationship be-

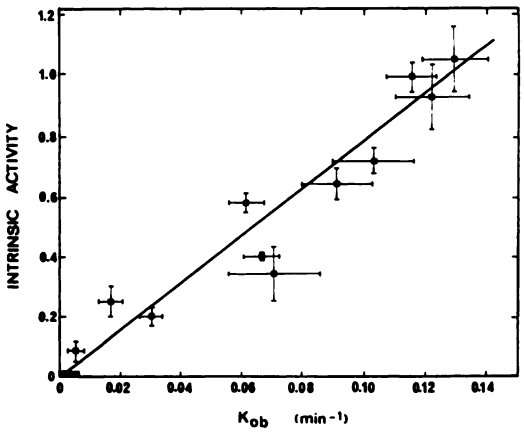

FIG. 7. *Rate of inactivation of (−)-^{3}H-DHA binding sites by NEM in presence of β-adrenergic agents in function of their intrinsic activity for adenylate cyclase activation*

The values for intrinsic activity and k_{ob} (pseudo-first-order rate constant for inactivation) are listed in Table 2. Each symbol represents a compound. The correlation coefficient $r = 0.97$.

tween this process and agonist-induced adenylate cyclase activation.

The first of these aspects was studied by the kinetic experiments shown in Figs. 1 and 2. The apparent first-order rate constant for inactivation (k_{ob}) was proportional to the concentration of NEM and to the fraction of sensitive receptors occupied by the agonists (Fig. 3). Accordingly, inactivation can be ascribed to a bimolecular reaction, which, because of the large excess of NEM (>5.5 μM) over the concentration of agonist-bound receptor sites (<0.6 nM), follows pseudo-first-order kinetics. The simplest explanation for these data is that after fast equilibrium binding of the agonists to the β-adrenergic receptors, a well-determined subpopulation of the binding sites becomes sensitive to inactivation by NEM, probably by alkylation (23). The opposite sequence of interactions (i.e., association of NEM with the receptor, followed by agonist-induced inactivation) is unlikely on the basis of data showing that treatment of the membranes with agonists after exposure to NEM and washing did not cause inactivation (18).

It is generally assumed that binding of an agonist molecule "induces" or "favors" a conformational transition of the receptor, to produce a stimulus with, in this case, adenylate cyclase activation as a measurable response. The observation that the β-adrenergic receptors of turkey erythrocyte membranes become sensitive to inactivation by NEM upon binding of agonist molecules, provides strong evidence for this assumption. In molecular terms, this increased sensitivity can be explained by the hypothesis that "essential" alkylable groups are normally embedded in the receptor molecule, and become exposed to NEM upon binding of the agonist.

Displacement-binding studies (Fig. 5) enable the direct evaluation of the equilibrium dissociation constant (K_D) of β-adrenergic ligands for binding to their receptor, and hence a study of the relationship between the number of occupied receptors and the response. The data (Table 2) indicate that for each ligand, half-maximal receptor occupation (K_D) and cyclase activation occur at a similar concentration. This observation, along with the fact that the Hill coefficient for cyclase activation is close to one (e.g., n_H = 0.97 and 1.01 for (−)- and (+)-epinephrine, respectively, from data presented in Fig. 3B), indicate that there is a proportional relationship between the amount of ocupied receptors and adenylate cyclase activation in our system. The proportionality constant is, however, distinct for each ligand. At full receptor occupation, various β-adrenergic ligands cause different levels of cyclase activation (Figs. 4, 5). This can be explained by the ability of the ligands to cause different conformational modifications of the receptor, resulting in different degrees of cyclase activation. Alternatively, it can be envisaged that the receptors can only adopt two conformations, one active and the other inactive, so that the fraction of occupied receptors assuming the active conformation is dependent on the nature of the ligand.

A choice between these explanations is made possible by comparing the ability of β-adrenergic ligands to cause adenylate cyclase activation and inactivation of the receptors by NEM. Since both phenomena are likely to be different nonlinear functions of the degree of conformational change of the receptor, they should be directly proportional to one another in case of a quantal transition between two states of the receptor, while no apparent relationship should exist in case of a gradual conformational change. The results (Fig. 7) plead for a quantal transition by showing a direct proportional relationship between the ability of the ligands to cause adenylate cyclase activation (measured by cyclase activation at full receptor occupancy) and inactivation of the receptors by a constant concentration of NEM (measured by k_{ob} at half-maximal receptor occupancy). According to the above considerations, cyclase activation should be directly proportional to the number of active receptors. Maximal cyclase activation is therefore a measure of the "intrinsic activity" of the ligand, i.e., the fraction of receptor occupations that give receptor activation (24).

The difference in intrinsic activity of the various ligands can be interpreted by at least two models.

Several authors have proposed an allosteric model (25–27), based on the Monod–Wyman–Changeux "plausible model" (28), schematically

$$\begin{array}{ccc} H + R_i & \overset{L}{\rightleftharpoons} & H + R_a \\ K_i \Downarrow\Uparrow & & \Downarrow\Uparrow K_a \\ H\cdot R_i & & H\cdot R_a \end{array} \qquad [5]$$

In this model, the receptor can exist in two forms, the active (R_a) and the inactive (R_i), to which the ligand (H) can bind with the microscopic equilibrium dissociation constants K_a and K_i, respectively. Both forms are in equilibrium, even in the absence of ligand. L is the equilibrium constant for the transition between the two forms of the receptor, i.e., $L = |R_i| / |R_a|$. Accordingly, ligands can act as agonists or antagonists on the basis of their selective affinity for either R_a or R_i.

For turkey erythrocyte membranes, the rate of inactivation of the β-adrenergic receptors by NEM and adenylate cyclase activity are very low in the absence of ligand. It can therefore be deduced that $L >> 1$, so that cyclase activation as well as inactivation of the β-receptors by NEM is in a high degree proportional to $|H\cdot R_a|$ (26). The macroscopic equilibrium dissociation constant (K_D) for binding and adenylate cyclase activation is by approximation (29)

$$K_D = (1/K_i + 1/L\cdot K_a)^{-1}. \qquad [6]$$

The intrinsic activity is proportional to the maximal amount of active sites (25),

$$[H\cdot R_a]_{max} = [R_t]/(1 + L\cdot K_a/K_i), \text{ when } [H] \to \infty, \qquad [7]$$

where $[R_t]$ is the total amount of receptor sites. For the turkey erythrocyte system, this model thus allows the interpretation of the binding and adenylate cyclase activation data as if the hormone-receptor interaction were a simple, reversible, bimolecular equilibrium binding process (26) given by Eq. [3].

However, if one assumes that the ligand induces a conformational change of receptor (30), it is possible to explain the data presented in this report equally well. The classical occupancy theory can be extended by assuming that the bound ligand induces an equilibrium

between the inactive and active state of the receptor. Schematically

$$H + R_i \overset{K_i}{\rightleftharpoons} H \cdot R_i \overset{L_H}{\rightleftharpoons} H \cdot R_a. \quad [8]$$

K_i is the microscopic equilibrium dissociation constant for binding of the ligand (H) to the inactive receptor (R_i), and L_H is the equilibrium constant for the transition between the inactive complex ($H \cdot R_i$) and the active complex ($H \cdot R_a$), i.e., $L_H = [H \cdot R_i]/[H \cdot R_a]$. In this model, the functional role of the ligand is to reduce the difference in free energy between the two conformations of the receptor; L_H is then a function of the considered ligand on the basis of the difference in free energy between $H \cdot R_a$ and $H \cdot R_i$. It can be demonstrated that for this model, the cyclase activation is always proportional to the amount of occupied sites. The macroscopic equilibrium dissociation constant (K_D) and the maximal amount of active sites are defined by Eqs. [9] and [10], respectively,

$$K_D = K_i \cdot L_H/(L_H + 1), \quad [9]$$

$$[H \cdot R_a]_{max} = [R_t]/(L_H + 1). \quad [10]$$

The main difference between these two models is that in the first one, both forms of the receptor are in equilibrium, even in the absence of ligand, whereas in the latter, the hormone induces a conformational change of the receptor. For both models, the macroscopic equilibrium dissociation constant (K_D) can still be determined as the concentration of the ligand that causes half-maximal binding or cyclase activation. However, this parameter has lost its original physical meaning, since it is related to the microscopic constants by either Eq. [6] or [9].

For both models, inactivation of β-adrenergic receptors by NEM can be described by the scheme

$$H \cdot R_a + NEM \xrightarrow{k'_2} r, \quad [11]$$

where k'_2 is the microscopic second-order rate constant. The macroscopic rate constant (k_2), defined by Eq. [2], equals $k'_2 \cdot [H \cdot R_a]_{max}/[R_t]$, for each ligand, and is thus proportional to the intrinsic activity (see Eqs. [7] and [10]).

The results presented in this report extend the already observed chemical differences between the β_1-adrenergic receptors in turkey erythrocyte membranes and the β_2-receptors in frog erythrocytes (10, 14). Whereas β-adrenergic agonists alone do not cause a decrease in the amount of (−)-^{3}H-DHA binding sites in turkey erythrocytes, they sensitize the receptor sites to inactivation by NEM. For the β_2-adrenergic receptors, agonists have been shown to cause a marked decrease in the (−)-^{3}H-DHA binding activity, i.e., "desensitization" (4), probably by inducing a high-affinity state of the receptor (17). This process is readily reversed by NEM (14, 17).

The desensitization phenomenon and the inactivation of agonist-bound receptors by NEM share several properties. Both processes affect only 40 to 60% of the receptor sites, and their rate is directly proportional to the intrinsic activity of the tested ligand (14). These similarities suggest that, although both receptors apparently have a different structure, their organization in the membrane may be equally affected by binding of agonist molecules.

In conclusion, two models have been proposed to explain the large variety of intrinsic activities of β-adrenergic ligands. Both models are also in accord with the observed inactivation of agonist-bound receptors by NEM (Eq. [4]), and with the close correlation between the rate constant of the inactivation process (k_2) and the intrinsic activity.

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