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THE β-ADRENERGIC RECEPTOR OF TURKEY ERYTHROCYTE MEMBRANES : CONFORMATIONAL MODIFICATION BY β-ADRENERGIC AGONISTS

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SUMMARY. The β-adrenergic receptors of turkey erythrocyte membranes have been identified by the specific binding of the radiolabeled antagonist (-)-|^{3}H|-dihydroalprenolol. Pretreatment of these membranes with either the alkylating agent N-ethylmaleimide or with β-adrenergic agonists does not affect (-)-|^{3}H| - dihydroalprenolol binding to the receptor sites. However, the simultaneous presence of both types of products causes a 50 % decline in the number of binding sites. A less pronounced decline occurs when the membranes are pretreated with N-ethylmaleimide in presence of the partial agonist (-)- phenylephrine, and no decline in the presence of the antagonist (-)-|^{3}H|-dihydroalprenolol. β -adrenergic agonists thus appear to induce a conformational change of their receptor, with results in an increased susceptibility to inactivation by N-ethylmaleimide.

Adenylate cyclase activation by catecholamines requires several steps : the specific binding of the hormone to β-adrenergic receptors on the cell membrane, the transmission of the signal to the enzyme and finally an increase in cyclic AMP production. The recent availability of radiolabeled β-adrenergic ligands such as (-)-|^{3}H|- dihydroalprenolol enables a direct characterization of the β-adrenergic receptors by binding studies.(1,2) Whereas occupation of these β-receptors by catecholamines and analogs can easily be determined by this method (3,4), the agonist or antagonist properties are usually studied indirectly by determining the ability to induce adenylate cyclase activation in cell membranes(3,5) or by evaluating the physiological responses in tissues (6). In this report, we show that β-adre-

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& Nonstandard abbreviations : |^{3}H|- DHA, (-)-|^{3}H|-dihydroalprenolol; NEM, N-ethylmaleimide.

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nergic receptors of turkey erythrocyte membranes can be rapidly inactivated by the reagent N-ethylmaleimide in the presence of agonists, but not of antagonists, providing for a new direct method to distinguish between these two types of compounds and confirming that they interact with the β-adrenergic receptors in a different way.

EXPERIMENTAL PROCEDURE. MATERIALS. The following were obtained as gifts : (-)-isoproterenol bitartrate (Sterling Winthrop) and phentolamine (Ciba-Geigy). (-)-Phenylephrine was purchased from Sigma Chemical Co.; pyrocatechol and N-ethylmaleimide were from Fluka. AG.(-)-|^{3}H|-dihydroalprenolol (33 Ci/ mmol) was obtained from New England Nuclear Corp.

Preparation of turkey erythrocyte membranes : Erythrocyte membranes were prepared according to Øye and Sutherland (7), suspended in 10 mM Tris-HCl (pH 7.4)/145 mM NaCl/2 mM $MgCl_2$ containing 10 % (vol/vol) glycerol, and stored in liquid nitrogen until use. Nucleated ghosts were obtained by hemolysis of erythrocytes in 5 mM Tris-HCl(pH 7.4)/2 mM $MgCl_2$. Protein determinations were performed by the procedure of Lowry et al(8) usingbovine serum albumin as the standard.

Membrane pretreatment with N-ethylmaleimide (NEM) & : Ghosts or membranes were preincubated with NEM and washed as follows :
Preincubation : 2 mg of membrane protein was preincubated with the indicated concentrations of NEM and adrenergic ligands at 30°C for 12 min. in 75 mM Tris-HCl(pH 7.4)/25 mM $MgCl_2$ (buffer A) in a final volume of 1 ml. Then, the reaction medium was immediately transferred into a 1.5 ml. Eppendorf micro test tube and centrifugated for 1 min in an Eppendorf centrifuge (1200 rpm) at 20°C.
Washing : After removal of the supernatant, the preincubated membranes were resuspended into 1 ml. of buffer A, and centrifugated for 1 min. This washing step was repeated once.

Binding of (-)-|^{3}H|- DHA & to pretreated ghosts or membranes was performed as published elsewhere (9). In all figures and tables, bound (-)-|^{3}H|- DHA refers to specific binding : i.e. binding of tracer displaced by 25 µM (±)-alprenolol. Data are mean at least 6 measurements, standard deviation less than 6 %.

Ascending thin-layer chromatography : Equal volumes of 40 mM (-)-isoproterenol and 40 mM NEM (in buffer A) were mixed, and incubated for 12 min. at 30°C. 5 µl of the (-)-isoproterenol and NEM solutions and 10 µl of the (-)-isoproterenol plus NEM solution were spotted on the same silicagel thin-layer chromatographic plate (20 x 20 cm, pre-coated, from Merck). After elution with ethyl acetate/formic acid/water (17/2/1), plates were exposed to iodine vapors. Isoproterenol : brown spot, R_F = 0.28; NEM : no colour; isoproterenol plus NEM : brown spot : R_F = 0.28.

RESULTS . The radiolabeled antagonist (-)-|^{3}H|- DHA has been shown to bind to the β_1-adrenergic receptors of turkey erythrocytemembranes with high specificity and affinity, and with fast association and dissociation kinetics at 30°C (9). Binding occurs to a single class of non-cooperative sites with an equilibrium dissociation constant (K_D) of 8 to 12 nM. As shown in figure 1, bound (-)-|^{3}H|- DHA is completely displaced by β-adrenergic agonists (with the order of potencies : (-)-isoproterenol ≃ (±)-proto-

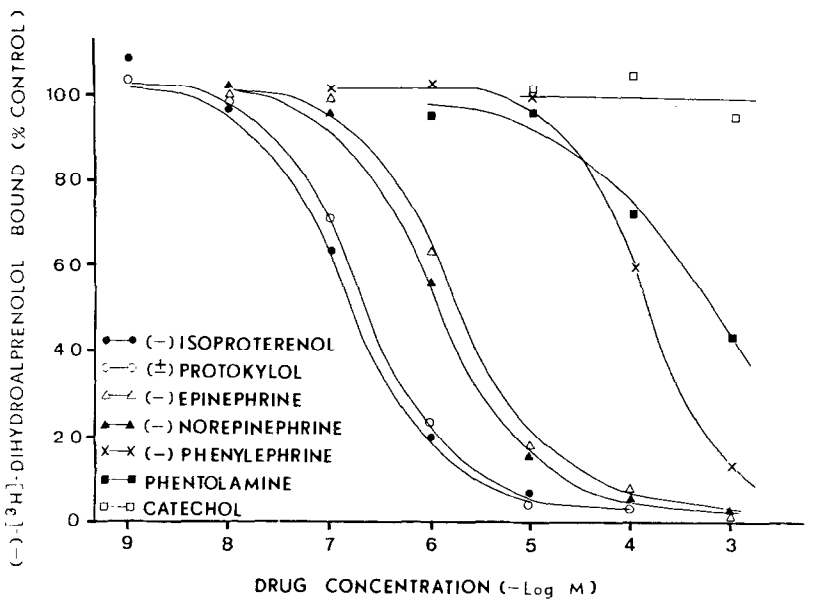

Fig.1. Competition of various drugs with (-)-|^{3}H|- DHA for binding to the β-adrenergic receptor of turkey erythrocyte membranes. Membranes (2 mg/ml) are incubated with 10 nM of (-)-|^{3}H|- DHA for 10 min. at 30°C in the presence of increasing concentrations of drug. Control binding refers to binding of (-)-|^{3}H|- DHA in the absence of competitor (0.11 pmol/mg protein).

kylol > (-)-norepinephrine ≃ (-)-epinephrine, typical for a β_1-adrenergic receptor), and by the partial agonist (-)-phenylephrine. The α-blocker phentolamine and the non-bioactive catechol produces weak or no displacement.

In contrast with the β-adrenergic receptors of a variety of tissues, the β_1-receptor of turkey erythrocyte membranes does not show desensitization upon prolonged exposure to β_1-adrenergic agonists (Table 1) (10-13). Binding of (-)-|^{3}H|- DHA to the β -adrenergic receptors is not affected by prior treatment of the membranes with NEM either (Table 1 and 2). However, pretreatment of the membranes with NEM in the presence of (-)-isoproterenol produces a 55 % drop in (-)-|^{3}H|- DHA binding (Table 1). A Scatchard plot of (-)-|^{3}H|- DHA saturation binding experiments, reveals that this drop is due to a decline in the number of receptor sites, and that the affinity of the tracer for the remaining receptors is not affected (unpublished observations). The number of inactivated receptors is not affected by improved washing of the NEM plus (-)-isoproterenol-treated membranes (i.e. washing followed by incubation with buffer and a second washing, instead of washing only, Table 1). Inactivation is apparently

Table 1: (-)-$|^3H|$- DHA binding to membranes after treatment with (-)-isoproterenol and/or NEM.

Membrane preincubation with: First preincubation	Second preincubation	(-)-$|^3H|$- DHA bound (% control)
– [a]	(-)-isoproterenol	103
–	NEM	103
–	(-)-isoproterenol + NEM	45
(-)-isoproterenol + NEM	–	44
(-)-isoproterenol	NEM	90
NEM	(-)-isoproterenol	95

Membranes (2 mg/ml) are preincubated with the indicated compounds ((-)-isoproterenol : 0.1 µM, NEM : 0.1 mM) for 12 minutes at 30°C, washed twice with buffer, preincubated for a second time at the same concentration with the indicated compounds, and washed twice with buffer. Binding of 10 nM (-)-$|^3H|$- DHA is measured. Control binding refers to binding to membranes treated with buffer only.
a) buffer only

Table 2: Effect of various drugs in presence or absence of NEM on (-)-$|^3H|$-DHA binding.

Membranes preincubated with:	(-)-$|^3H|$- DHA binding (% control) to: no NEM	NEM : 10 mM
buffer only	100	98
(-)-isoproterenol : 0.1 µM (agonist , K_D = 0.12 µM [a])	93	53
(-)-phenylephrine : 0.1 mM (partial agonist , K_D = 0.094 mM)	94	81
(-)-$|^3H|$- DHA : 10 nM (antagonist , K_D = 10 nM)	105	92
phentolamine : 1 mM (α-antagonist , K_D = 0.26 mM)	104	98
catechol : 1 mM (non-bioactive , K_D > 0.5 mM)	98	93

Membranes (2 mg/ml) are pretreated with various drugs in the presence or absence of NEM for 12 min. at 30°C, washed twice with buffer and incubated with 10 nM (-)-$|^3H|$- DHA. Control binding refers to binding to membranes pretreated with buffer only.
a) K_D-values were determined as previousely described (9).

irreversible. Neither treatment of the membranes with NEM followed by washing and subsequent treatment with (-)-isoproterenol, or the reverse treatment, causes a pronounced reduction in (-)-$|^3H|$- DHA binding (Table 1). Therefore, receptors are only inactivated by NEM and (-)-isoproterenol when both compounds are simultaneously present.

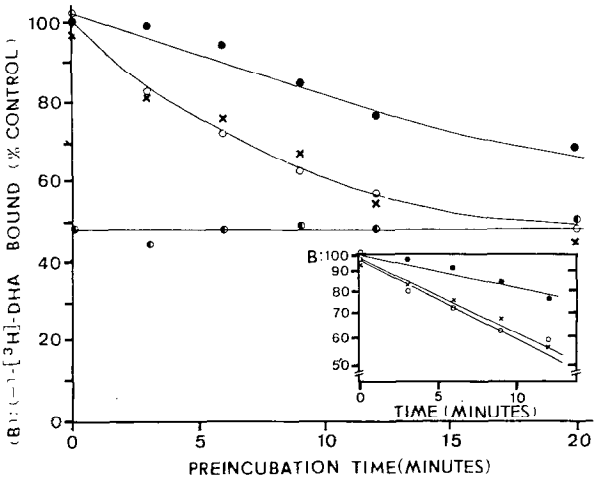

Fig.2. Inactivation of β-adrenergic receptors by NEM plus (-)-isoproterenol : time dependence. Membranes (2 mg/ml) are pretreated with 0.025 µM (-)-isoproterenol plus NEM (50 µM : (●—●) , 0.1 mM : (o—o) , 1 mM : (⊙—⊙)), or with 0.05 µM (-)-isoproterenol plus 50 µM NEM : (x—x) , for different periods of time at 30°C. Then membranes are washed twice with buffer and binding of 10 nM (-)-|^{3}H|- DHA measured.
Insert : Semi-logarithmic representation. (B) is binding to membranes pretreated with NEM plus (-)-isoproterenol for the time (t). The apparent first-order rate constant of inactivation (k_{ob}) is defined by equation : $\ln (B/100) = - k_{ob}.t$.

The effect of NEM plus (-)-isoproterenol is time- and dose- dependent. The kinetic data, presented in Fig. 2, indicate that pretreatment of turkey erythrocyte membranes with 50 µM NEM plus 0.025 µM (-)- isoproterenol produces a time - dependent decrease in the number of (-)-|^{3}H|- DHA binding sites. When the number of binding sites is plotted on a logarithmic scale (Fig. 2, inset), the decrease appears to be related linearly to the preincubation time. The apparent first-order rate constant for the inactivation (k_{ob}) approximates 0.022 min^{-1}. The rate of inactivation increases about twice by doubling the concentration of either (-)-isoproterenol (k_{ob} = 0.046 min^{-1}) or NEM (k_{ob} = 0.044 min^{-1}).

Pretreatment of the membranes with (-)-isoproterenol (0.025 µM) in the presence of a high concentration of NEM (1 mM), causes inactivation of about 50 % of the receptor sites within less than 1 min (i.e. the time required for a first washing of the membranes, as described in the experimental procedure). After this rapid decline, the amount of receptor sites remains constant upon prolonging the preincubation for at least 15 min (Fig. 2). Higher concentrations of (-)-isoproterenol (0.1 µM) and NEM (10 mM) do not

affect the remaining receptors in either membranes or nucleated ghosts. After 5, 10 and 15 min preincubation, the (-)-|^{3}H|- DHA binding activity is still respectively 54, 48, and 48 % for membranes, and 52, 52 and 57 % for nucleated ghosts. Accordingly, the concentration of NEM or (-)-isoproterenol or the membrane purfication are not the limiting factors for the extent of inactivation.

Inactivation of the β-adrenergic receptors by NEM plus (-)- isoproterenol is in part mimicked by NEM in the presence of the partial agonist (-)-phenylephrine, but not in the presence of the antagonist (-)-|^{3}H|- DHA (Table 2). The concentrations of the β-adrenergic ligands used in table 2, were equal to their K_D-value for the receptor, so that the three compounds tested occupied 50 % of the receptor sites. Under the same preincubation conditions, NEM plus either the α-antagonist phentolamine or the non-bioactive catechol do not affect (-)-|^{3}H|- DHA binding to the membranes.

DISCUSSION : Although the alkylating agent NEM inactivates various types of hormone receptors (14,15) and β-adrenergic agonists cause desensitization of their specific receptors in several tissues (10-12), neither of these two types of compounds independently affect the β_1-adrenergic receptors of turkey erythrocyte membranes. However, there is a marked decrease in the number of the receptors (as defined by (-)-|^{3}H|- DHA binding sites) when both the reagent and the agonist (-)-isoproterenol are present simultaneously (Table 1). As a chemical modification of the (-)-isoproterenol molecule by NEM has not been observed by thin-layer chromatography (Materials and Methods), it is likely that these compounds inactivate the β-adrenergic receptor in a synergistic fashion. Binding of agonists to the β_1-adrenergic receptors of turkey erythrocyte membranes may cause a conformational change, resulting in an increased sensitivity of the receptors towards alkylation (inactivation) by NEM. Both processes can be summarized by the following scheme :

$$H + R \rightleftharpoons H.R' \xrightarrow{NEM} r$$

where H is the agonist; R, H.R' and r the free, agonist-bound and inactivated receptor respectively. The irreversible step in this set of reactions (Table 1) is probably due to the formation of covalent bonds between NEM and the receptors. The kinetic data (Fig. 2) agree with the proposed scheme by showing a linear relationship between the rate of inactivation and the concentration of both NEM and (-)-isoproterenol (at submaximal concentrations, where $[HR'] \simeq [H]$). The lack of decline in (-)-|^{3}H|- DHA binding activity after consecutive treatment of the membranes with the reagent and the agonist (Table 1), may be explained by the reversible interaction between β-adrenergic agonists and receptors.

There are some striking similarities between agonist-induced conformational changes of the β-receptors and adenylate cyclase activation, suggesting that both phenomena are closely related : i) Both processes are fast and reversible (Fig. 2, Table 1 and ref. 16) ii) The ability of β-adrenergic ligands to induce adenylate cyclase activation (intrinsic activity) is closely related to their ability to sensitize the β-adrenergic receptors to inactivation by NEM. Inactivation is more pronounced in presence of the agonist (-)-isoproterenol than in presence of the partial agonist (-)-phenylephrine, and not significant in presence of the antagonist (-)-|^{3}H|-DHA (Table 2 and ref. 5)

Recently, Cassel and Selinger have reported that the transmission of information between the β-receptors and adenylate cyclase in turkey erythrocytes involves the activation of a membrane-bound GTP-ase(17). The catecholamine-stimulated GTP-ase activity was more sensitive to NEM in presence of (-)-isoproterenol than in its absence. The inactivation of the β-adrenergic receptor by NEM in the presence of β-adrenergic agonists might explain this observation.

Although the β-adrenergic ligands interact with all the (-)-|^{3}H|- DHA binding sites(Fig.1), NEM inactivates no more than half in the presence of (-)-isoproterenol(Fig. 2). The existence of two equally represented categories of β-adrenergic receptors, of two binding sites on a single receptor molecule, or the dissociation of binary receptor-complexes by agonists might be considered as possible interpretations of the partial inactivation. At present, we can not conclude which moiety of the β-receptor is alkylated by NEM. This reagent can form covalent bonds with sulphydryl-,amino- or imidazole groups(18).

We observed recently that solubilization selectively increases the affinity of β-adrenergic receptors for agonists but not for antagonists(9). This fact argued for a different type of molecular interaction between the receptor and both types of ligands. The present results suggest that a change of conformation is induced by the binding of agonists but not of antagonists to the β-adrenergic receptor, as indicated by the increased sensitivity to inactivation by NEM. The relationship between the conformational change of the receptor and the adenylate cyclase activation, is now under investigation.

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