Biochemical Pharmacology, Vol. 38, No. 17, pp. 2755–2761, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

USE OF *N*-ETHYLMALEIMIDE TO DIRECTLY DETERMINE THE PROPORTION OF β-ADRENERGIC RECEPTORS EXHIBITING HIGH AFFINITY FOR AGONISTS ON HUMAN MONONUCLEAR LEUKOCYTE MEMBRANES*

JAMES W. RIGBY and ALASTAIR J. J. WOOD
Vanderbilt University School of Medicine, Department of Pharmacology, Division of Clinical Pharmacology, Nashville, TN 37232, U.S.A.

(*Received* 11 *May* 1988; *accepted* 20 *December* 1988)

Abstract—Following incubation with the β-adrenergic agonist isoproterenol (ISO) and the alkylating agent *N*-ethylmaleimide (NEM), the number of β-adrenergic receptors measured on human mononuclear leukocyte (MNL) membranes decreased. Pretreatment induced an approximately 40% loss of measurable receptors. The proportion of receptors lost was that which formed the ternary complex of agonist–receptor–G_s protein (H–R–G_s) and exhibited high affinity for agonists. In comparison studies, the loss of receptors was found to be similar to the proportion of receptors that form the high affinity state of the receptor ($\%R_H$) for agonist in agonist competition binding studies. Acute exposure to agonist induced a similar reduction in ISO/NEM "sensitive" receptors and the $\%R_H$. These experiments indicate that pretreatment of MNL membranes with ISO/NEM is a powerful tool with which to directly determine the percentage of receptors able to form an agonist high affinity state.

Recent studies have shown that stimulation of adenylate cyclase by catecholamines or β-adrenergic agonists involves the interaction of at least three separate proteins: the β-adrenergic receptor itself, a stimulatory guanine nucleotide-sensitive regulatory protein, and the catalytic enzyme [1–3]. It has been proposed that, in the sequence of molecular events, the binding of agonist (H) to the receptor (R) promotes the coupling of this agonist–receptor complex (HR) to the stimulatory regulatory guanine nucleotide binding protein (G_s) to form a ternary complex of H–R–G_s [4–7]. Binding of guanine nucleotides to G_s dissociates the ternary complex and results in the stimulation of the catalytic protein by activated G_s and the subsequent formation of cAMP [8, 9].

The formation of the ternary complex is a necessary intermediate in the stimulation of adenylate cyclase [7–9]. The ternary complex displays high affinity for agonists [4, 6], and we and others have measured previously the proportion of receptors in the high affinity state indirectly by computer modeling of agonist binding in competition with a radiolabeled antagonist [10–12]. In the absence of guanine nucleotides, the competition curves are "shallow," with slopes of less than one, and can be resolved into both high and low affinity states of the receptor for agonist [13, 14]. The addition of guanine nucleotides shifts the competition curve to the right and reduces the affinity of the receptors for agonist which results in a homogeneous population of low affinity receptors and a slope of "normal" steepness [8, 13].

It has been recognized recently that a proportion of the β-adrenergic receptor population is lost following incubation with agonists and an alkylating agent, such as *N*-ethylmaleimide [15–23]. It is suggested that the coupling of the HR complex to G_s, to form the H–R–G_s complex, exposes sulfhydryl groups which are attacked by the alkylating agent to cause a "locking-in" of the agonist into a slowly-dissociating ternary complex. These studies, therefore, suggested that the population of receptors "sensitive" to attack with an agonist and an alkylating agent shall be those that form the high affinity complex for agonist in competition binding studies.

We have shown recently, using computer analysis of agonist competition binding curves, that there is a loss of high affinity receptors for the agonist isoproterenol (ISO) in hypertensives and the elderly on mononuclear leukocyte (MNL) membranes [24, 25]. Although this approach has provided important insights into the mechanisms responsible for altered β-receptor responsiveness it is both time consuming and expensive. The purpose of this present study was to determine (1) if loss of β-adrenergic receptors on human mononuclear leukocytes occurred when they were incubated with agonist and the alkylating agent NEM, (2) whether the loss of receptors matched those exhibiting high affinity for agonist, and (3) whether this procedure could be used as a simpler alternative to agonist competition binding to quantify directly the proportion of receptors exhibiting high affinity for agonists.

MATERIALS AND METHODS

Materials. (−)-Isoproterenol-(+)-bitartrate, *N*-ethylmaleimide, 5′-guanylimidodiphosphate (GppNHp) and *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) were purchased from the Sigma Chemical Co. (St Louis, MO). (−)-[^{125}I]Iodopindolol (2200 Ci/mmol) was purchased from the New England Nuclear Corp. (Boston, MA).

* Supported by USPHS Grants HL14192, AG01395 and RR95.

† Address reprint requests to A. J. J. Wood, M.D.

All other chemicals were of analytical grade.

Membrane preparation. A modification of the method of Feldman *et al.* [10] was used to prepare MNL membranes. Briefly, whole blood was drawn from normal healthy individuals and added to pre-chilled polypropylene tubes containing 0.14% (w/v) EDTA. After removal of the platelet-rich plasma, the MNL (80–90% lymphocytes) were isolated according to the method of Boyum [26]. Following washing and centrifugation, the MNL pellet was resuspended in ice-cold 2 mM Tris–HCl (pH 7.4) containing 3 mM EDTA, homogenized for 10 sec (Brinkmann Polytron; setting 7) and centrifuged for 10 min at 38,000 *g* at 4°. The final membrane pellet was resuspended in Buffer A containing 20 mM HEPES (pH 7.4), 100 mM NaCl, 25 mM $MgCl_2$ and 3 mM EDTA.

Pretreatment of MNL membranes with isoproterenol and N-ethylmaleimide. Membranes (0.8 ml) were prewarmed for 5 min at 30° then incubated with 0.5 μM ISO for 10 min and then for another 10 min following the addition of 0.5 mM NEM in a final volume of 1 ml of Buffer A. Membranes were incubated in Buffer A alone as a control. The pretreatment phase was terminated by the addition of 4 ml of ice-cold buffer containing 20 mM HEPES, 100 mM NaCl and 3 mM EDTA (Buffer B), followed by centrifugation at 38,000 *g* at 4° for 10 min. The membranes were washed another two times before resuspension in Buffer B.

[^{125}I]Iodopindolol (I-PIN) binding assay. The binding of I-PIN was to a single population of binding sites of high affinity and was saturable. The binding of I-PIN was displaced by other ligands stereospecifically and with the potency appropriate for a β_2-adrenergic receptor. The washed membranes were incubated in triplicate, with 100 pM I-PIN for 10 min in Buffer B, containing 4 μg/ml bovine serum albumin and 0.5 mM ascorbic acid, at 37° in a total volume of 250 μl. The binding reaction was stopped by the addition of 10 ml Buffer B and rapidly filtered through pre-soaked Whatman GF/C filters. Filters were then washed with an additional 10 ml buffer, and the radioactivity retained on the filters was determined in a Beckman Gamma counter model 5500 at 70% efficiency. Specific binding was calculated as the difference in the amount of I-PIN bound in the absence and presence of ISO (0.1 mM) and was routinely 75–95% of total binding.

Agonist competition binding studies. In the agonist competition binding studies, control and ISO/NEM pretreated membranes were incubated with I-PIN and ISO (0.1 nM to 0.1 mM) in the absence and presence of GppNHp as previously described [10]. Competition curves were analyzed by non-linear weighted regression analysis, using the computer program LIGAND [27]. Binding curves were fitted to a one or a two affinity state model with points weighted to the inverse of the square of the variance. Total and non-specific binding were determined by the overall plateaus of the binding curves and did not differ significantly from the values obtained empirically. The fit of the data to a one or two affinity state model was compared by F test, and the two state affinity model was accepted with $P < 0.05$. For the two affinity state model, estimates were provided by the computer of the association constants for the high (K_H) and low (K_L) affinity states and the percentage of receptors in the high affinity state ($\%R_H$). The IC_{50}, defined as the concentration of agonist required to reduce binding by 50%, and slope values were determined using the four-parameter logistic equation as described by DeLean *et al.* [28].

Protein concentration was determined by the method of Lowry *et al.* [29], using bovine serum albumin as a standard.

Data in the figures and tables refer to the mean ± SE of the specific binding of I-PIN.

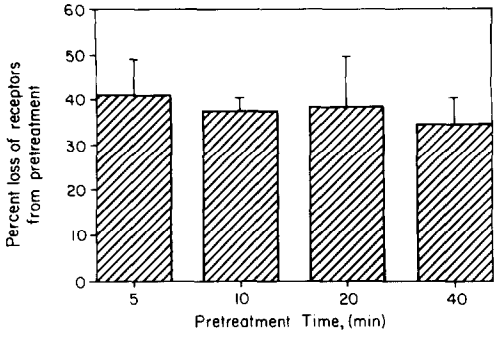

Fig. 1. Loss of I-PIN β-adrenergic receptor binding after ISO/NEM pretreatment. MNL membranes were incubated with ISO (0.5 μM) and NEM (0.5 mM) at 30° for the indicated periods of time, washed three times, and assayed for I-PIN binding. Incubation with buffer alone (control binding) was constant throughout the duration of the experiment. Values are means ± SE, N = 0.

RESULTS

Pretreatment of MNL membranes with ISO (0.5 μM) and NEM (0.5 mM) resulted in the rapid loss of approximately 40% of identifiable β-adrenergic receptors after 5 min. No further loss of receptors occurred if the pretreatment step was prolonged for up to 40 min (Fig. 1). Over this same time period control and non-specific binding was constant. Since maximal loss of receptors was evident within 10 min, this time period was used in subsequent experiments to determine the percentage of ISO/NEM "sensitive" receptors (see Materials and Methods).

The specificity of this receptor loss with ISO/NEM pretreatment is shown in Fig. 2. Loss of receptors only occurred when membranes were incubated in the presence of both ISO and NEM. No significant change in β-adrenergic receptor density occurred when membranes were incubated with ISO or NEM alone which was 103 and 94% of control binding respectively. Figure 2 also shows that the addition of (−)-pindolol (1 μM) to the incubation, before addition of NEM, prevented the loss of receptors induced by ISO/NEM pretreatment. Thus, loss of β-adrenergic receptors on MNL membranes only occurred when both isoproterenol and NEM were present, did not occur in response to either of these when added alone, and was blocked by preincubation with the β-receptor antagonist pindolol.

An alternative explanation for the loss of β-

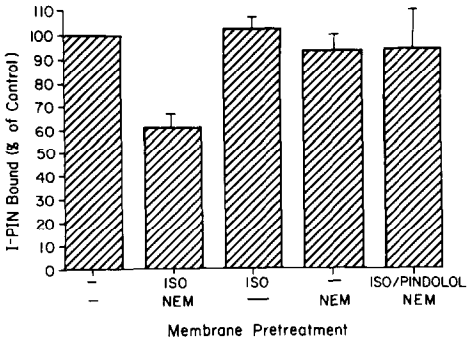

Fig. 2. Effect of pretreatment with ISO and/or NEM on I-PIN binding to MNL membranes. Membranes were incubated with ISO (0.5 μM), and/or NEM (0.5 mM) and/or (−)-pindolol (1 μM), as indicated for 10 min at 30°, washed, and assayed for I-PIN binding. Control binding (100%) was in the presence of buffer alone. Data are means ± SE of three experiments.

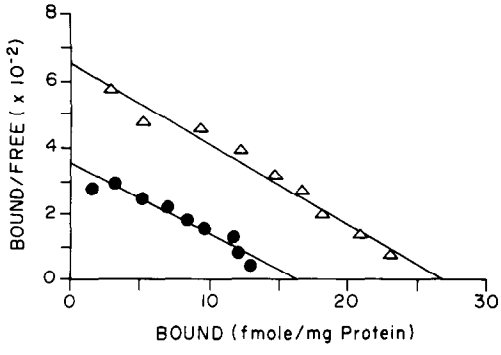

Fig. 3. Scatchard plot of specific I-PIN binding to human MNL membranes. Membranes were incubated with ISO (0.5 μM)/NEM (0.5 mM) or buffer alone as a control for 10 min at 30°. Following washing, membranes were incubated with I-PIN (5–300 pM) for 10 min at 37°. Binding was analyzed by non-linear regression analysis and I-PIN bound to a single high affinity binding site. Control membranes (△): B_{max} = 25.4 fmol/mg protein, K_d = 33.6 pM); ISO/NEM pretreated membranes (●) (B_{max} = 15.2 fmol/mg protein, K_d = 37.0 pM). Data are from one of four separate experiments which gave similar results.

adrenergic receptors, identifiable with a single concentration of I-PIN, might have been that the incubation with ISO/NEM altered the affinity of the receptors for I-PIN. To exclude this possibility, control and pretreated membranes were incubated with a range of I-PIN concentrations (5–300 pM) as previously described (see Materials and Methods). As shown in Fig. 3, ISO/NEM pretreatment resulted in a marked reduction in receptor density but did not alter the affinity of the receptors for I-PIN.

To determine which β-adrenergic receptors on MNL membranes were "sensitive" to ISO/NEM pretreatment, control and ISO/NEM pretreated membranes were resuspended in Buffer A and incubated with I-PIN (100 pM) and ISO (0.1 nM to 0.1 mM) for 10 min at 37°. In the absence of GppNHp, the binding of ISO to β-adrenergic receptors in control

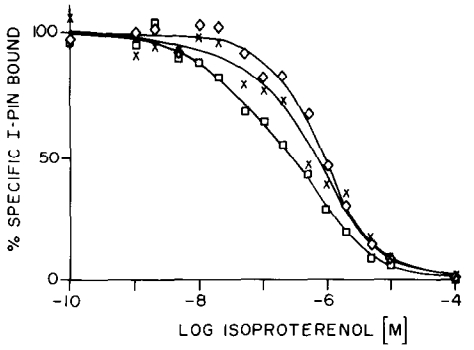

Fig. 4. ISO/I-PIN competition curves on MNL membranes. Membranes were pretreated with ISO (0.5 μM)/NEM (0.5 mM) for 10 min at 30°. Control membranes were incubated in buffer alone. Membranes were resuspended in Buffer A and incubated with 100 pM I-PIN and increasing concentrations of ISO for 10 min at 37°. Control membranes, no GppNHp: (K_H = 2.5 nM, K_L = 131 nM and $\%R_H$ = 39.0) (□); with GppNHp (0.1 mM): (one site fit K_L = 145 nM) (◇) ISO/NEM pretreated membranes: (K_H = 3.0 nM, K_L = 155 nM, and $\%R_H$ = 15.2) (×). Data are from one of three separate experiments. In this experiment, 49% of the receptors were lost after ISO/NEM pretreatment.

membranes was best described by a two affinity state model of high and low affinity state receptors for agonist and a $\%R_H$ of 36.3 ± 2.4%. Addition of GppNHp shifted the agonist competition curve to the right and resulted in the curve being best described by a one-affinity state model having a single low affinity for agonist (Fig. 4). The change in affinity induced by GppNHp resulted in an increase in IC_{50} from 0.34 to 0.84 μM (Table 1) and in the slope from −0.75 to −0.96. When compared with control membranes, pretreatment with ISO/NEM resulted in a shift of the isoproterenol competition curve to the right, increasing the IC_{50} to 0.55 μM, and a reduction in the measurable $\%R_H$ to 11.45 ± 5.8%. In addition, ISO/NEM decreased the number of measurable β-adrenergic receptors by 51.6%. When the reduction of receptors was taken into consideration, the concentration of high affinity receptors remaining after pretreatment was 1.44 ± 0.77 fmol/mg protein which, when compared with the concentration of high affinity receptors in the control membranes of 9.13 ± 1.13 fmol/mg protein, was equivalent to an 85% loss of receptors able to bind agonist with high affinity. Thus, pretreatment of MNL membranes with ISO/NEM results in the selective loss of those receptors that form the high affinity ternary complex.

Since β-adrenergic receptors "sensitive" to ISO/NEM pretreatment are those that form a high affinity complex with agonist, we assessed whether pretreatment with ISO/NEM could be used as a simpler alternative to agonist competition binding studies to determine the $\%R_H$. Membranes were either pretreated with ISO/NEM and assayed with a single concentration of I-PIN, as described in Materials and Methods, or incubated with I-PIN (20 pM) and ISO (0.1 nM to 0.1 mM) for 60 min at 37° in the presence and absence of GppNHp as previously described

Table 1. Effect of ISO/NEM pretreatment on ISO/I-PIN competition binding

	IC_{50} (μM)	K_H (nM)	K_L (mM)	$\%R_H$	Concentration high affinity receptors (fmol/mg protein)
Control					
−GppNHp	0.34 ± 0.07	6.20 ± 1.73	145 ± 11	36.3 ± 2.4	9.13 ± 1.13
+GppNHp	0.84 ± 0.04		140 ± 3		
Treated	0.55 ± 0.08	2.67 ± 0.33*	146 ± 9*	11.4 ± 5.8	1.44 ± 0.77

After incubation with ISO/NEM or buffer alone as a control for 10 min at 30°, membranes were incubated with 100 pM I-PIN and increasing concentrations of ISO for 10 min at 37° (see Fig. 4). Pretreatment resulted in a 51.6% decrease in I-PIN binding. GppNHp = 5′-guanylimidodiphosphate. Values are means ± SE of three separate experiments, except where noted.

* Average of two experiments. One experiment was best described by one affinity state model.

[8]. In these parallel experiments, the percentage of receptors "sensitive" to ISO/NEM pretreatment was 43 ± 2.9% which was not significantly different from the $\%R_H$ of 46 ± 2.5% determined from agonist competition binding studies. Therefore, these preliminary experiments suggested that ISO/NEM pretreatment was a good predictor of the $\%R_H$ determined in competition binding studies.

To extend these findings, we assessed the effects of prior incubation with ISO on both the percentage of receptors "sensitive" to ISO/NEM pretreatment and the $\%R_H$. Whole blood was incubated with either ISO (10 μM) in ascorbic acid (1 mM) or in ascorbic acid alone, as a control, before membrane preparation. This procedure did not alter the affinity of I-PIN for the receptor or significantly affect receptor density. As expected, preincubation with ISO resulted in a shift to the right of the subsequent isoproterenol competition binding curve, increasing the IC_{50} from 103 ± 8 to 142 ± 12 nM and the slope from −0.74 ± 0.02 to −0.82 ± 0.04 but did not alter the estimates for the high and low affinity states of the β-adrenergic receptor (Fig. 5A). The $\%R_H$ was reduced from 35.9 ± 2.3 to 19.1 ± 5.1% which was similar to the reduction in the proportion of receptors "sensitive" to ISO/NEM which decreased from 39.3 ± 4.2 to 14.9 ± 4.6% (Fig. 5B). The relationship between the estimates of $\%R_H$ and proportion of receptors "sensitive" to ISO/NEM determined in control and ISO pretreated membranes is shown in Fig. 6.

DISCUSSION

The stimulation of adenylate cyclase by β-adrenergic agonists to produce cAMP involves a number of molecular events within the membrane. Crucial in this process of transmembrane signaling is the formation of a ternary complex of H–R–G_s protein. This complex displays high affinity for agonists, and we have previously indirectly quantified the percentage of receptors exhibiting the high affinity state for agonist ($\%R_H$) on MNL membranes using agonist competition for a radiolabeled antagonist [8, 21, 22].

It has been shown in various tissues and cell types that exposure of β-adrenergic receptors to agonists in the presence of alkylating agents [i.e., *N*-ethylmaleimide, 2,2′-dinitro-5,5′-dithiodibenzoic acid (DTNB)] results in the loss of a proportion of the receptor population [15–23]. The loss is agonist specific since it is not induced by antagonists and it involves other membrane components as well as the receptor since loss of receptors is absent in those cell types lacking a G_s-protein [17] and can be prevented by solubilization of the receptor or by addition of guanine nucleotides [17, 20, 23]. These studies, therefore, infer that the loss of receptors is the result of an agonist-induced conformational change in a proportion of the receptors which "sensitizes" them to attack by alkylating agents. It is likely that this induced change involves the formation of the ternary complex with the G_s-protein. Several mechanistic models have been proposed to account for the loss in identifiable receptors. A common feature of these models is that sulfhydryl groups are exposed following the formation of the ternary complex. Although the exact site of these groups is not known, recent evidence indicates that the sulfhydryl groups are present on the G_s-protein itself [23]. It is thought that the formation of the ternary complex (H–R–G_s) exposes previously hidden sulfhydryl groups within the G_s-protein which are then covalently modified by the alkylating agent to form a stable agonist–receptor complex, and thus subsequent binding of other ligands to the receptor is prevented.

Pretreatment of human MNL membranes with ISO/NEM resulted in a rapid but incomplete loss of approximately 40% of the measurable β_2-adrenergic population. This finding is consistent with previous studies in other tissues which have shown that only a proportion of the β-adrenergic receptor population was lost following incubation with agonist and alkylating agents. The loss of β-adrenergic receptors from MNL membranes was agonist specific since no receptor loss was observed in the presence of NEM alone and was prevented by the simultaneous presence of an antagonist. Also, a change in the affinity of the receptor for the radioligand could not explain the reduction in receptor number.

A subsequent experiment indicated to us that the measured loss of β-adrenergic receptors was the result of the formation of a "persistent" ternary complex. Binding of ISO to control MNL membranes, in the absence of GppNHp, was best

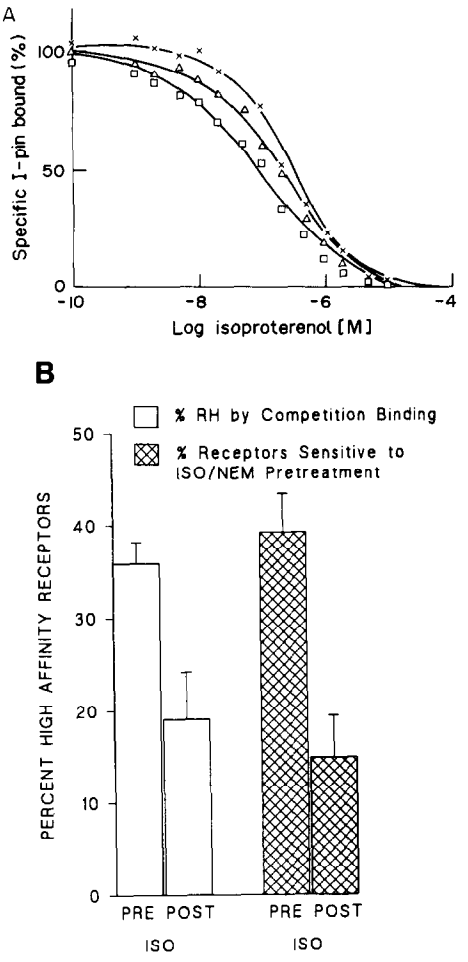

Fig. 5. Effect of prior exposure to ISO (10 μM) on isoproterenol competition curves (A) and on the estimates of $\%R_H$ and the percentage of ISO/NEM "sensitive" receptors. (B). Aliquots of blood were incubated with ISO (10 μM) for 15 min at 37° before MNL membrane preparation. Blood was incubated with ascorbic acid as a control. (A) ISO/I-PIN competition curves on MNL membranes. Control and ISO treated membranes were incubated with 20 pM I-PIN and increasing concentrations of ISO for 60 min at 37° in Tris buffer as previously described [10]. Control. (□—□): no GppNHp (K_H = 4.8 nM, K_L = 122 nM $\%R_H$ = 36.0); with 0.1 mM GppNHp (×· · ·×) (one site fit K_L = 127 nM); ISO treated membranes (△—△): no GppNHp (K_H = 2.0 nM, K_L = 106 nM, $\%R_H$ = 19.0). Binding in the presence of GppNHp after ISO pretreatment was superimpossible with control GppNHp; therefore it is not shown (one site fit K_L = 121 nM). Data are from a typical experiment (N = 8). (B) Comparison of the $\%R_H$ measured by competition binding (left-hand columns) and percentage of receptors sensitive to ISO/NEM pretreatment (right-hand columns) pre and post preincubation with ISO (10 μM).

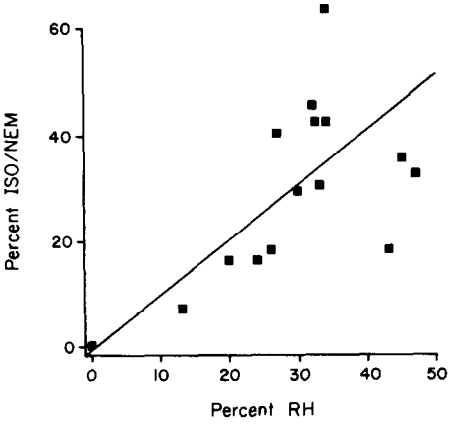

Fig. 6. Comparison of $\%R_H$ obtained from competition binding experiments and the percent of receptors "sensitive" to ISO/NEM pretreatment. Points used are from the ISO pretreatment experiments and represent the individual data obtained in control membranes and in membranes with ISO (10 μM).

described by a two affinity state model of high and low affinity state receptors, confirming the results of previous studies [10, 24, 25]. Addition of GppNHp resulted in a decrease in affinity for ISO through the conversion to low affinity and a reduction in the percentage of high affinity receptors. ISO/NEM pretreatment also resulted in a decrease in the affinity of the agonist ISO for β-adrenergic receptors. This decrease in the percentage of high affinity state receptors and reduction in receptor affinity for agonist suggest that the receptor population which is lost to the I-PIN binding after ISO/NEM incubation is that population of the receptors exhibiting high affinity for agonist (R_H) and which is represented by the high affinity ternary complex.

In these experiments it was also observed that ISO/NEM pretreatment did not remove completely the population of β-adrenergic receptors that could form a high affinity complex since only 85% of the original high affinity receptor population was lost. This incomplete loss may be the result of receptor reactivation occurring during the short incubation period to determine the $\%R_H$. Receptor "reactivation" following membrane pretreatment has been shown in previous studies which have shown that receptors are reactivated when pretreated membranes are exposed to magnesium ions, guanine nucleotides, and high pH [20, 23]. However, it is well documented that magnesium ions are an absolute requirement for β-adrenergic receptors to express high affinity state for agonists [21, 30]. Thus, it is likely that the small percentage of receptors displaying high affinity for agonist after ISO/NEM pretreatment is the result of receptor reactivation. This finding lends support to the evidence indicating that the site of action of NEM is on the G_s-protein and not on the receptor recognition site itself.

The finding that β-adrenergic receptors on MNL membranes were "sensitive" to ISO/NEM pretreatment and that it was selective for those receptors that could form a high affinity state complex led us to hypothesize that the ISO/NEM procedure might be utilized as a direct assay for the proportion of high affinity state receptors for agonist on human MNL membranes. To evaluate this hypothesis we performed parallel studies comparing the proportion of receptors "sensitive" to ISO/NEM pretreatment and the $\%R_H$ determined from agonist competition

binding studies. These studies showed that the proportion of receptors no longer measured after ISO/NEM incubation of leukocytes from humans was similar to the $\%R_H$ determined on leukocyte membranes from the same individuals. This finding prompted us to assess the applicability of the method of quantify changes in the proportion of high affinity state receptors following *in vitro* modulation of the $\%R_H$ with ISO. Studies have shown that acute exposure of β-adrenergic receptors on MNL membranes to agonists results in the loss of high affinity receptors and a functional uncoupling from adenylate cyclase both *in vitro* and *in vivo* [10, 31, 32]. Thus, we proposed that short exposure of the MNL membranes to ISO *in vitro* should cause a reduction in receptors exhibiting high affinity for agonists. The loss in $\%R_H$ would be paralleled by a similar reduction in receptors "sensitive" to ISO/NEM pretreatment. Comparing individual values revealed a significant positive correlation between the $\%R_H$ and the proportion of ISO/NEM "sensitive" receptors (Fig. 6), implying that the proportion of receptors exhibiting high affinity for agonist was modulated similarly to those lost by exposure to ISO/NEM. It is of interest to note that the proportion of receptors binding agonist with high affinity and those sensitive to ISO/NEM pretreatment was substantially less than 100%; this is in keeping with previous findings [10, 24, 25] and presumably reflects the rate of reassociation of receptor–G-protein complexes.

In conclusion, β_2-adrenergic receptors on human MNL membranes have been shown to be "sensitive" to pretreatment with an agonist and alkylating agents. It is evident that the population of receptors lost during this procedure are those receptors that can form the high affinity ternary complex of H–R–G_s. Several recent studies have alluded to the importance of the high affinity receptor state in humans, and a reduction in the percentage of these receptors has been shown in the elderly and in hypertensives [24, 25]. Pretreatment with ISO/NEM has obvious advantages over the conventionally used agonist competition binding assays to measure changes in the $\%R_H$. First, it provides a direct measure of the percentage of high affinity state receptors for agonist. Second, it is a simpler procedure and lends itself to the measurement of a large number of samples simultaneously. Finally, it is a direct measure not subject to the interpretative difficulties of computer curve fitting. Mechanisms involved in the regulation of β-adrenergic receptors in humans are still under investigation, and the use of pretreatment with ISO/NEM is an additional tool which will assist in determining the factors altering β-receptor responsiveness in humans.

REFERENCES

1. Ross EM and Gilman AG, Biochemical properties of hormone-sensitive adenylate cyclase. *Annu Rev Biochem* **49**: 533–564, 1980.
2. Rodbel M, The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature (Lond)* **284**: 17–22, 1980.
3. Gilman AG, G proteins and dual control of adenylate cyclase. *Cell* **36**: 577–579, 1984.
4. May DC, Ross EM, Gilman AG and Smigel MD, Reconstitution of catecholamine-stimulated adenylate cyclase activity using three purified proteins. *J Biol Chem* **260**: 15829–15833, 1985.
5. DeLean A, Stadel JM and Lefkowitz RJ, A ternary complex explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J Biol Chem* **255**: 7108–7117, 1980.
6. Cerione RA, Codina J, Benovic JL, Lefkowitz RJ, Birbaumer L and Caron MG, The mammalian β_2-adrenergic receptor: reconstitution of functional interactions between pure receptor and pure stimulatory nucleotide binding protein of the adenylate cyclase system. *Biochemistry* **23**: 4519–4525, 1984.
7. Limbird LE, Gill DM and Lefkowtiz RJ, Agonist-promoted coupling of the β-adrenergic receptor with the guanine nucleotide regulatory protein of the adenylate cyclase system. *Proc Natl Acad Sci USA* **77**: 775–779, 1980.
8. Stadel JM, DeLean A and Lefkowitz RJ, A high affinity agonist beta adrenergic receptor complex is an intermediate for catecholamine stimulation of adenylate cyclase in turkey and frog erythrocyte membranes. *J Biol Chem* **255**: 1436–1441, 1980.
9. Lefkowitz RJ, Caron MG, Michel T and Stadel JM, Mechanisms of hormone receptor–effector coupling: the β-adrenergic receptor and adenylate cyclase. *Fed Proc* **41**: 2664–2670, 1982.
10. Feldman RD, Limbird LE, Nadeau J, FitzGerald GA, Robertson D and Wood AJJ, Dynamic regulation of leukocyte beta adrenergic receptor–agonist interactions by physiological changes in circulating catecholamines. *J Clin Invest* **72**: 164–170, 1983.
11. Samuelson WM and Davies AO, Hydrocortisone-induced reversal of beta-adrenergic receptor uncoupling. *Am Rev Respir Dis* **130**: 1023–1026, 1984.
12. Brodde O-E, Brinkmann M, Schemuth R, O'Hara N and Daul A, Terbutaline-induced desensitization of human lymphocyte β_2-adrenoceptors. Accelerated restoration of β-adrenoceptor responsiveness by prednisone and ketotifen. *J Clin Invest* **76**: 1096–1101, 1985.
13. Kent RS, DeLean A and Lefkowiz RJ, A quantitative analysis of beta-adrenergic receptor interactions: resolution of high and low affinity states of the receptor by computer modeling of ligand binding data. *Mol Pharmacol* **17**: 14–23, 1985.
14. Hancock AA, DeLean A and Lefkowitz RJ, Quantitative resolution of beta-adrenergic receptor subtypes by selective ligand binding: application of a computerized model fitting technique. *Mol Pharmacol* **16**: 1–9, 1980.
15. Bottari S, Vauguelin G, Durieu O, Klutchko C and Strosberg AD, The β-adrenergic receptor of turkey erythrocyte membranes: conformational modification of β-adrenergic agonists. *Biochem Biophys Res Commun* **86**: 1311–1318, 1979.
16. Vauquelin G, Bottari S and Strosberg AD, Inactivation of β-adrenergic receptors by *N*-ethylmaleimide: permissive role of β-adrenergic agents in relation to adenylate cyclase. *Mol Pharmacol* **17**: 163–171, 1980.
17. Vauquelin G and Maguire M, Inactivation of β-adrenergic receptors by *N*-ethylmaleimide in S49 lymphoma cells. Agonist induction of functional receptor heterogeneity. *Mol Pharmacol* **18**: 363–369, 1980.
18. Jacobsson B, Vauquelin G, Wesslau G, Smith U and Strosberg AD, Distinction between two subpopulations of β_1-adrenergic receptors in human adipose tissue. *Eur J Biochem* **114**: 349–354, 1981.
19. Vauquelin G, Bottari S, Andre C, Jacobsson B and Strosberg AD, Interaction between β-adrenergic receptor and guanine nucleotide sites in turkey membranes. *Proc Natl Acad Sci USA* **77**: 3801–3805, 1980.

20. Korner M, Gilon C and Schramm M, Locking of hormone in the β-adrenergic receptor by attack on a sulfhydryl in an associated component. *J Biol Chem* **257**: 3389–3396, 1982.
21. Heidenreich KA, Weiland GA and Molinoff PB, Effects of magnesium and *N*-ethylmaleimide on the binding of ^{3}H-hydroxybenzylisoproterenol to β-adrenergic receptors. *J Biol Chem* **257**: 804–810, 1982.
22. Andre C, Vauquelin G, Severne Y, DeBacker J-P and Strosberg AD, Dual effect of *N*-ethylmaleimide on agonist-mediated conformational changes of β-adrenergic receptor. *Biochem Pharmacol* **31**: 3657–3662, 1982.
23. Severne Y, Wemers C and Vauquelin G, Agonist/*N*-ethylmaleimide mediated inactivation of β-adrenergic receptors. *Biochem Pharmacol* **34**: 1611–1617, 1985.
24. Feldman RD, Limbird LE, Nadeau J, Robertson D and Wood AJJ, Leukocyte β-receptor alterations in hypertensive subjects. *J Clin Invest* **73**: 648–653, 1984.
25. Feldman RD, Limbird LE, Nadeau J, Robertson D and Wood AJJ, Alterations in leukocyte beta-receptor affinity with aging. A potential explanation for altered beta-adrenergic sensitivity in the elderly. *N Engl J Med* **310**: 815–819, 1984.
26. Boyum A, Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* **21** (Suppl 97): 77–89, 1968.
27. Munson PJ and Rodbard D, LIGAND: A versatile computerized approach for characterization of ligand-binding systems. *Anal Biochem* **107**: 220–239, 1980.
28. DeLean A, Munson PJ and Rodbard D, Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose–response curves. *Am J Phyisol* **235**: E97–E102, 1978.
29. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurements with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
30. Williams LT, Mullikin D and Lefkowitz RJ, Magnesium dependence of agonist binding to adenylate cyclase-coupled hormone receptors. *J Biol Chem* **253**: 2984–2989, 1978.
31. De Blasi A, Lipartiti M, Moltulsky HJ, Insel PA and Fratelli M, Agonist-induced redistribution of β-adrenergic receptors on intact human mononuclear leukocyte redistributed receptors are nonfunctional. *J Clin Endocrinol Metab* **61**: 1081–1088, 1985.
32. Motulsky HJ, Cunningham EMS, DeBlasi A and Insel PA, Desensitization and redistribution of β-adrenergic receptors on human mononuclear leukocytes. *Am J Physiol* **250**: E583–E590, 1986.