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Agonist-Induced Changes in the Properties of *Beta*-Adrenergic Receptors on Intact S49 Lymphoma Cells

Time-Dependent Changes in the Affinity of the Receptor for Agonists

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SUMMARY

The binding of the antagonist [^{125}I]iodopindolol to *beta*-adrenergic receptors on intact wild-type S49 mouse lymphoma cells and mutants that have impaired abilities to generate cyclic AMP in response to catecholamines was studied. The binding of [^{125}I]iodopindolol is of high affinity (K_D = 35 pM), rapid, stable over 90 min, and rapidly reversible ($t_{1/2}$ = 8 to 11 min). Nonspecific binding was very low (<5% of total binding at the K_D). Kinetic and competition experiments performed under steady-state and non-equilibrium conditions revealed that the binding characteristics for agonists were very different in intact cells and in membranes. The interactions of antagonists, on the other hand, appeared to be identical in studies carried out with intact cells and membranes. In intact cells, the affinity of the receptor for agonists was observed to decrease rapidly within the first 5 min of exposure of the cells to an agonist. Competition experiments revealed that at least 80% of the receptor-agonist complex was in a high-affinity state when studies were carried out using short incubation times (0.5–1 min). Under equilibrium conditions, about 80% of the complex in wild-type, uncoupled, and kinase-deficient cells was of a low affinity. At equilibrium, only low-affinity binding was seen with coupling protein-deficient cells. This rapid, time-dependent decrease in the affinity of receptors for agonists was seen with most agonists although not with zinterol. The phenomenon was not due to differences in the kinetics of the interactions of agonists and [^{125}I]iodopindolol with the receptor, and it is likely that the receptor undergoes a conformational change upon exposure to agonists. This effect was not observed in membranes and was not related to the presence of a functional guanine nucleotide-binding protein or to the production of cyclic AMP. Furthermore, hydrophilic agonists and antagonists, under short-term incubation conditions, did not fully compete for the binding sites labeled with the lipophilic radioligand [^{125}I]iodopindolol, although this binding was fully and stereospecifically competed for by lipophilic antagonists. This suggests that in untreated cells a small but significant fraction of the receptors is sequestered in an environment not accessible to hydrophilic ligands.

INTRODUCTION

The development of radioligands with high specific radioactivity that bind to receptors with high affinity has led in the past 8 years to a large number of publications dealing with results of studies of the binding of radioligands to *beta*-adrenergic receptors (1, 2). Most of this work was performed on membrane preparations from different organs and species. Although our knowledge of hormone-receptor interactions has been greatly increased as a result of these studies, a major concern is that receptors on membranes may have properties different from those of receptors on intact cells. Several groups (3–16) have presented data describing results obtained in experiments with living cells. Whether [^{125}I]IHYP[2] or [^{3}H]dihydroalprenolol was used as the radioligand, nonspecific binding as compared with total bind-

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[2] The abbreviations used are: [^{125}I]IHYP, (±)-[^{125}I]iodohydroxybenzylpindolol; [^{125}I]IPIN, (−)-[^{125}I]iodopindolol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; WT, wild-type S49 cells; *cyc*$^-$, coupling protein-deficient cells; *unc*, uncoupled cells; *kin*$^-$, kinase-deficient cells; *G/F*, guanine nucleotide-binding regulatory protein.

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ing was high. This was probably related to uptake of the radioligand that could be reversed by prolonged washing (6, 7, 13) or the use of drugs such as chloroquine (11). A common feature of these studies was the discrepancy observed between the affinity of the receptor for agonists in cells and in membranes. The affinities of receptors for agonists in membrane preparations appeared to be 1 to 3 orders of magnitude higher than the affinities determined in intact cells. Recently, Pittman and Molinoff (15) presented data suggesting that the affinity of receptors for agonists in intact L6 muscle cells decreased rapidly in the presence of agonists.

The purpose of the present study was to document the decrease in the affinity of *beta*-adrenergic receptors for agonists in intact cells by analysis of the results of experiments performed under equilibrium and non-equilibrium conditions. [^{125}I]IPIN was chosen as the radioligand because it is associated with relatively small amounts of nonspecific binding (13). Since it was of interest to determine whether changes in affinity are related to uncoupling mechanisms and/or cyclic AMP-mediated events, these experiments were carried out with S49 lymphoma cells, including mutants that have lesions in the catecholamine-stimulated adenylate cyclase system.

In this paper evidence is presented that lipophilic ligands such as [^{125}I]IPIN are suitable for binding studies in intact cells. Furthermore, uptake of these ligands does not contribute to the observed binding. Nonspecific binding of these ligands in intact cells is comparable to that observed in membrane preparations under identical incubation and washing conditions. A rapid, marked decrease in the affinity of receptors for agonists was observed in S49 lymphoma cells. The effect was not a result of cyclic AMP production or of uncoupling of the receptor from a functional guanine nucleotide-binding protein. The results may be due to an agonist-induced conformational change in the receptor.

Finally, evidence is presented that approximately 20% of the *beta*-adrenergic receptors in intact S49 cells are sequestered in an environment not accessible to hydrophilic ligands. This population of receptors can be detected only at very short incubation times. Thus, non-equilibrium binding studies provide a powerful experimental approach for the investigation of the properties of receptors on intact cells (16).

METHODS

Iodination procedure. (−)-Pindolol was iodinated as previously described. Pindolol (20 μg), dissolved in 20 μl of 0.0135 M HCl, was mixed with 20 μl of 0.3 M KH_2PO_4 (pH 7.4), 20 μl of chloramine T (0.17 mg/ml), and 20–50 μl of Na^{125}I for 3 min at room temperature. The reaction was stopped by addition of 300 μl of $Na_2S_2O_3$ (1 mg/ml) and 10 μl of 1 N NaOH. The iodinated product was extracted three times into 300 μl of ethyl acetate containing 0.01% phenol. The extracts were combined and spotted on 3M chromatography paper. Descending chromatography was run for 4–6 hr at room temperature in 0.5 M ammonium formate (pH 8.5) containing 0.01% phenol. The chromatogram was cut into strips, and the ligand was extracted with methanol containing 0.01% phenol. The ligand (specific activity 2.2 Ci/μmole) was stored at −20°. Fresh ligand was prepared every 3 weeks.

Cell culture. Stock cultures of S49 lymphoma cells were maintained in Spinner culture at a density of 0.5 to 1.0 × 10^6 cells/ml in Dulbecco's modification of Eagle's medium supplemented with 10% horse serum in a humidified incubator containing 10% CO_2/90% air at 37°.

Membrane preparation for binding assays. Cells, grown in 8-liter Spinner flasks to a density of approximately 1 × 10^6 cells/ml, were harvested at room temperature, and purified plasma membranes were prepared from these cells according to the procedure of Ross *et al.* (17). The bands at the interface of the 30–40% and 20–30% layers were collected, diluted with HME buffer (20 mM Hepes (pH 7.4)/2 mM $MgCl_2$/1 mM EDTA), and centrifuged at 57,500 × *g* in an SW 50.2 rotor in a Beckman L8-70 ultracentrifuge. The pellets were resuspended in 30–40 ml of 20 mM Na-Hepes/0.9% NaCl (pH 7.4), homogenized with a Polytron homogenizer, and stored frozen in 2-ml aliquots at −70°. Membrane suspensions were thawed and homogenized with the Polytron prior to being used in binding assays.

Protein determinations were performed according to the method of Bradford (18), using BSA as a standard.

Binding studies with intact cells. Binding experiments with intact cells were carried out in polystyrene tubes (Sarstedt 55-463). Intact cells did not remain viable for extended periods of time in the polypropylene tubes used in studies with membranes. The final incubation volume was 250 μl, containing 50 μl of radioligand, 50 μl of competing drug or buffer, and 150 μl of cell suspension. Nonspecific binding was defined with 50 μM (−)-metoprolol. Equilibrium binding was usually performed for 45 min.

The cells, ligand, and competing drugs were made up in Leibowitz's L15 medium containing 2% horse serum, glucose (1 g/liter), and 5 mM Hepes (pH 7.6). Cells (400,000–900,000/150 μl) were suspended in this medium at 37° 1 hr before the experiment was started.

Assays were stopped by dilution with 10 ml of ice-cold buffer (10 mM Tris-HCl/154 mM NaCl, pH 7.5). Samples were then filtered over Schleicher and Schuell glass-fiber filters (No. 30) on a Millipore filtering device. The filters were washed with an additional 10 ml of buffer at room temperature and dried with suction. Radioactivity was determined in a Beckman 4000 *gamma* counter at an efficiency of 74%.

Binding studies with membranes. The protocol for binding studies with membranes was similar to that for intact cells, except that radioligand and competing drugs were made up in a mixture of ascorbate (25 mg/100 ml) and BSA (10 mg/100 ml). Membranes (2–5 μg of protein) were suspended in 20 mM Hepes (pH 7.6) containing 154 mM NaCl. For equilibrium studies, membranes, radioligand, and competing drugs were added to tubes on ice, and the reaction was initiated by placing the tubes in a 37° bath. For non-equilibrium studies, all components were preheated to 37° prior to initiating the assay.

Data analysis. Binding data were analyzed, as described earlier (19), by nonlinear least-squares curve fitting according to the law of mass action.

Competition experiments were analyzed according to the following equations:

$$B = C + \sum_{i=1}^{n} \frac{B_{\max_i}}{1 + \frac{K_D}{L}(1 + I/K_{I_i})}$$

where C is a constant (nonspecific binding) and K_D and L are the equilibrium dissociation constant and concentration of free radioligand. $B_{\max_i}$ is the maximal number of binding sites in state i, and I and K_{I_i} are the free concentration and the equilibrium dissociation constant of the competing ligand for the state i. Since many studies were performed under non-equilibrium conditions, competition experiments were also analyzed according to:

$$B = C + \sum_{i=1}^{n} \frac{\text{Bound}_i}{1 + I/\text{IC}_{50_i}}$$

where IC_{50_i} represents the concentration of competing ligand which inhibits 50% of the specific binding to receptors in state i, I is the concentration of competing drug, C is nonspecific binding, and Bound_i is [^{125}I]IPIN bound to state i.

Saturation experiments were analyzed according to:

$$B = \sum_{i=1}^{n} \frac{B_{max_i}}{1 + K_{D_i}/L}$$

where B_{max_i} is the maximal number of binding sites in state i, K_{D_i} is the equilibrium dissociation constant for the receptors in state i, and L is the concentration of free radioligand. Association and dissociation rate constants were estimated as described earlier (20). The dissociation rate constant k_{off} was estimated from the exponential plot $B_t = B_0 e^{(-k_{off} t)}$, where B_0 and B_t represent the amount of [^{125}I]IPIN bound at times 0 and t, respectively. The association rate constant k_{on} was estimated from the pseudo first-order plot $\ln(B_{eq} - B_t/B_{eq}) = k_{obs} \times t$, where B_{eq} and B_t represent the amount of [^{125}I]IPIN bound at equilibrium and time t, respectively; $k_{obs} = k_{on} - k_{off} \times$ [[^{125}I]IPIN].

Statistical analysis was performed according to the extra sum of squares principle (21), and the F-test was performed according to Snedecor and Cochran (22):

$$F = \frac{\frac{SS_1 - SS_2}{df_1 - df_2}}{\frac{SS_2}{df_2}}$$

where SS_1 and SS_2 are the sums of the squares of the residuals for the less and the more complicated systems (e.g., one state versus two states) and df_1 and df_2 are the degrees of freedom of the less and the more complicated systems.

The F-value was tested with $df_1 - df_2$ degrees of freedom for the numerator, and df_2 degrees of freedom for the denominator. Improvement of the fit was analyzed by comparing a two-state with a one-state model and then a three-state with a two-state model. The data were analyzed using interactive computer programs written in PL/PROPHET on a DEC10 computer using the PROPHET system (23). Nonlinear regression analysis was performed using MLAB (24), which is part of the PROPHET system.

Materials used. The following drugs were kindly provided as gifts: (−)-pindolol and (−)-cyanopindolol were from Dr. G. Engel, Sandoz Ltd. (Basel, Switzerland); Cc25 from Prof. M. Staehelin, Friedrich Miescher Institut (Basel); (−)- and (+)-propranolol from Ayerst Laboratories Inc. (New York, N. Y.); sotalol from Mead Johnson and Company (Indianapolis, Ind.); metoprolol from A. B. Hässle (Göteborg, Sweden); salbutamol from Allen and Hanbury's Research, Ltd. (Toronto, Ont., Canada). Isoproterenol and epinephrine were purchased from Sigma Chemical Company (St. Louis, Mo.).

RESULTS

Binding characteristics of [^{125}I]IPIN to beta-adrenergic receptors on intact S49 WT and cyc$^-$ cells. Saturation experiments were performed on intact S49 WT and *cyc*$^-$ cells (data not shown). Binding was saturable and of high affinity (B_{max} = 1850 and 2500 receptors/cell, K_D = 36 and 31 pM in WT and *cyc*$^-$ cells, respectively). Both nonlinear regression and Scatchard analysis of the data suggested that all of the receptors have the same affinity for the ligand. Nonspecific binding was low, about 5% of total binding at the K_D value and 12–14% of total binding at 5 times the K_D value. Similar experiments performed with iodocyanopindolol in WT cells gave similar results (data not shown). K_D values for [^{125}I]IPIN obtained in studies with membrane preparations were comparable (35–40 pM) to those obtained in studies with intact cells.

Binding of [^{125}I]IPIN under the conditions used in these studies was rapid in WT and *cyc*$^-$ cells (data not shown); the half-times of association were 10 and 7 min, respectively. Binding was fully reversible within 60 min; the half-times of dissociation were 14 min in WT cells and 10 min in *cyc*$^-$ cells. Association rate (k_{on}) values were 1.6 × 10^9 min^{-1} M^{-1} and 2 × 10^9 min^{-1} M^{-1}, and dissociation rate (k_{off}) values were 0.05 min^{-1} and 0.07 min^{-1} for WT and *cyc*$^-$ cells, respectively. The K_D values obtained from these kinetic experiments, 32 pM for WT cells and 35 pM for *cyc*$^-$ cells, were consistent with K_D values observed at equilibrium.

It was necessary to show that the binding of [^{125}I]IPIN is stereoselective, since lipophilic ligands may be taken up when experiments are carried out with intact cells (6, 11). (−)-Propranolol was 50–100 times more potent in inhibiting the binding of [^{125}I]IPIN than was (+)-propranolol (K_D = 0.3 and 32.4 nM for (−)- and (+)-propranolol, respectively). Both (−)- and (+)-propranolol fully inhibited specific binding of [^{125}I]IPIN without inhibiting nonspecific binding. Similar results were obtained with WT cells and *cyc*$^-$ cells (data not shown).

Time course of the binding of agonists and antagonists to intact cells and membrane preparations. The time course of the binding of [^{125}I]IPIN in WT and *cyc*$^-$ cells was determined in the presence and absence of isoproterenol (Fig. 1A and B). The presence of isoproterenol delayed [^{125}I]IPIN binding for a short time (*inset 1*, Fig. 1A and B), after which binding increased rapidly, reaching equilibrium at about the same time as did the binding of [^{125}I]IPIN in the absence of an agonist. In *cyc*$^-$ cells, the lowest concentration of isoproterenol used (0.5 μM) did not compete at equilibrium for binding sites labeled by [^{125}I]IPIN, whereas, in WT cells, about 15% of the binding was inhibited when assays were carried out in the presence of 0.5 μM isoproterenol. The amount of [^{125}I]IPIN bound in the presence of isoproterenol was expressed as a function of the amount of ligand bound in the absence of isoproterenol (*inset 2*, Fig. 1A and B). This transformation of the data revealed that there was a rapid decrease in the ability of isoproterenol to compete for [^{125}I]IPIN binding sites on WT and *cyc*$^-$ cells. Similar effects were observed when experiments were carried out with the agonists epinephrine and Cc25 (data not shown). The extent and the rapidity of the effects observed with these agonists were similar to those observed with isoproterenol.

The sigmoidal shape of the time courses observed in the presence of isoproterenol and the dramatic change seen when the data were transformed indicate that isoproterenol is not inhibiting the binding of [^{125}I]IPIN in a normal competitive manner. Two explanations of these findings were investigated: isoproterenol may be binding more rapidly to the receptors than does [^{125}I]IPIN, resulting in an overestimate of the initial capacity of isoproterenol to inhibit the binding of [^{125}I]IPIN, or the agonist may be inducing a decrease in the affinity of the receptors for agonists.

To investigate the possibility of a kinetic effect, experiments were carried out with membrane preparations from both WT and *cyc*$^-$ cells (Fig. 2). Isoproterenol inhibited the binding of [^{125}I]IPIN to receptors on membranes in a normal competitive fashion (*inset 1*, Fig. 2A and B). Transformation of the data, as in Fig. 1, showed

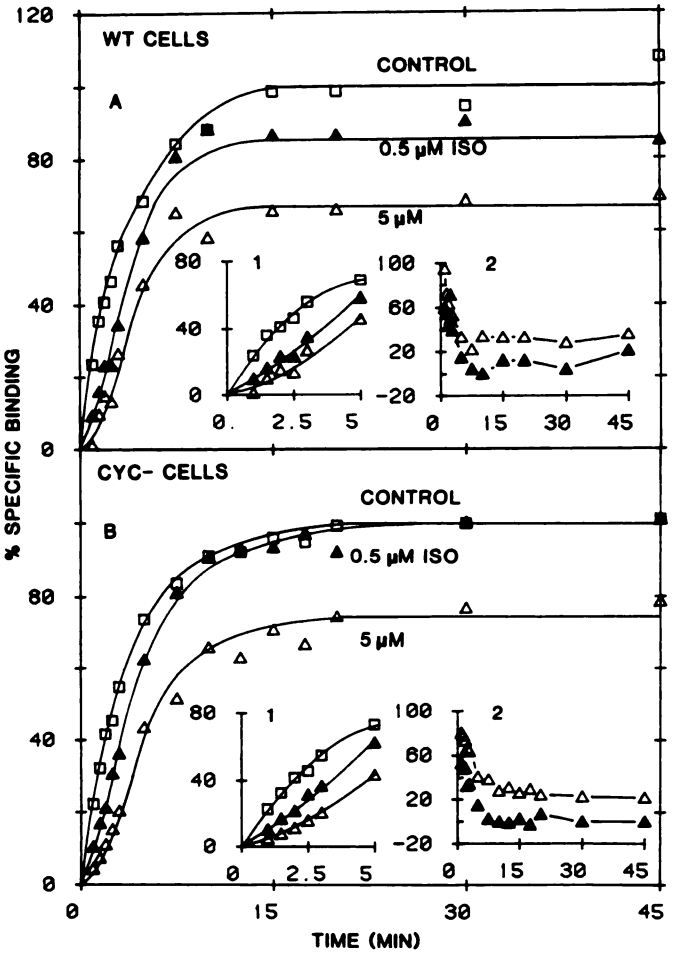

FIG. 1. *Time course of the binding of [^{125}I]IPIN to receptors on intact cells in the presence of isoproterenol*

WT cells (400,000/250 μl) (A) and *cyc*$^-$ cells (300,000/250 μl) (B) were incubated with [^{125}I]IPIN (186 and 114 pM, respectively) in the absence (□) and presence of 0.5 (▲) or 5.0 (△) μM isoproterenol for varying times between 1 and 45 min. Results are expressed as percentage of [^{125}I]IPIN specifically bound versus time (minutes), with 100% representing the amount specifically bound in the control experiment at equilibrium. *Inset 1* shows the data for the first 5 min. *Inset* 2 shows a transformation of the data wherein each point is expressed as percentage displacement in the presence of isoproterenol as compared with controls in which the binding of [^{125}I]IPIN was determined in the absence of isoproterenol. Total and nonspecific binding were determined in triplicate. Results are representative of eight and two similar experiments with WT and *cyc*$^-$ cells, respectively.

that the effect of isoproterenol was approximately constant throughout the time course of the assay (*inset 2*, Fig. 2A and B).

The phenomenon of rapid, agonist-induced changes in the affinity of *beta*-adrenergic receptors on intact cells for agonists has also been observed in L6 muscle cells (15). We have previously reported that the full agonists zinterol and salmefamol did not induce a decrease in the affinity of the receptor for agonists. In S49 WT cells, the affinity of the receptor for zinterol was the same in intact cells as on membranes (Fig. 3, *inset*).

In contrast to results obtained with isoproterenol in intact S49 cells, metoprolol (an antagonist) displayed strictly competitive behavior even under non-equilibrium conditions (Fig. 4A and B). The binding isotherms of [^{125}I]IPIN in the presence of metoprolol were typical of normal competition, and transformation of the data indicated that the ability of metoprolol to compete for binding sites for [^{125}I]IPIN was approximately constant. The same type of experiments performed with metoprolol in membranes yielded similar results (data not shown).

Competition experiments performed at different incubation times in WT and cyc$^-$ *cells and membranes.* Competition experiments were carried out at incubation times between 0.5 and 60 min. Cells or membranes were incubated with [^{125}I]IPIN and 16–19 concentrations of an agonist or antagonist. Since some of these experiments were performed at very short incubation times, the concentration of the radioligand was usually relatively high, between 3 and 6 times the K_D value. Cells were incubated for 1 min with [^{125}I]IPIN and varying concentrations of propranolol. The (−)-isomer of propranolol was approximately 30-fold more potent than the (+)-isomer even

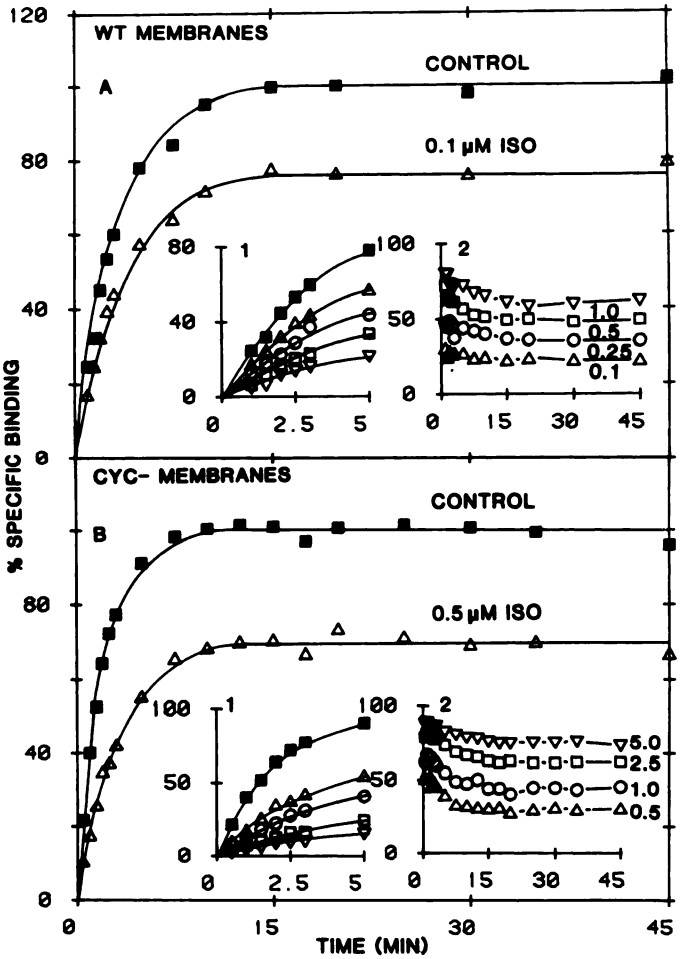

FIG. 2. *Time course of the binding of [^{125}I]IPIN to receptors on membranes in the presence of isoproterenol*

A. WT membranes (4 μg/250 μl) were incubated with [^{125}I]IPIN (105 pM) for varying times between 1 and 45 min in the absence (■) and presence of 0.1 (△), 0.25 (○), 0.50 (□), or 1.0 (▽) μM isoproterenol. B. *Cyc*$^-$ membranes (4 μg/250 μl) were incubated with [^{125}I]IPIN (159 pM) in the absence (■) or presence of 0.5 (△), 1.0 (○), 2.5 (□), or 5.0 (▽) μM isoproterenol. The *insets* represent the same transforms as in Fig. 1. Total and nonspecific binding were determined in triplicate.

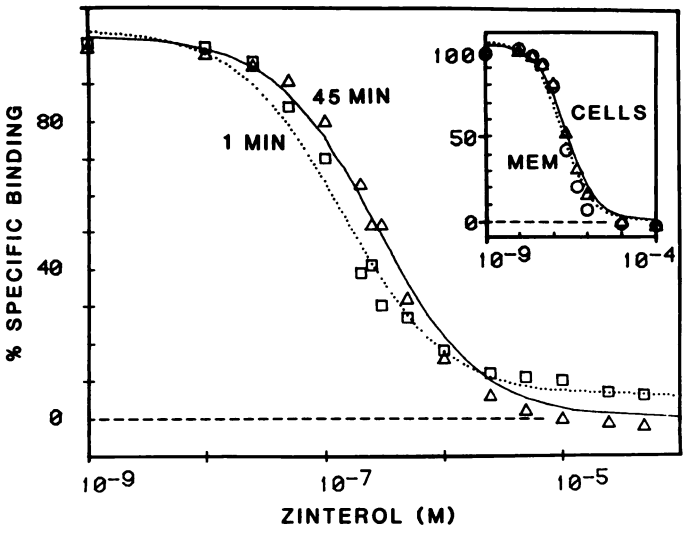

FIG. 3. *Inhibition of the binding of [^{125}I]IPIN to receptors on intact cells and membranes by zinterol*

WT cells (550,000–750,000/250 μl) were incubated for 1 min (□) or 45 min (△) with [^{125}I]IPIN (106–123 pM) and different concentrations of zinterol. *Inset.* WT cells (△) or membranes (○; 25–31 μg/250 μl) were incubated for 45 min with [^{125}I]IPIN and zinterol under similar conditions. The results are expressed as percentage of specifically bound [^{125}I]IPIN versus the concentration of zinterol. The lines are computer-fit to a single affinity state. The data points represent the average values obtained in three experiments. Each determination was carried out in triplicate.

under these non-equilibrium conditions. Similar results were obtained with WT (*inset*, Fig. 5A) and *cyc*$^-$ (*inset*, Fig. 5B) cells.

Competition experiments performed with the antagonist metoprolol in intact cells revealed only small changes between equilibrium and non-equilibrium conditions. The affinity of the receptor for this antagonist differed by a factor of 2–3 when 1- and 45-min competition experiments were analyzed (Fig. 5). The specific binding of [^{125}I]IPIN was fully inhibited by metoprolol, and data points best fitted a single-affinity state of the receptor. When competition experiments were performed with metoprolol in membranes (data not shown), the results were the same as those observed in studies carried out with intact cells. The affinity of the receptor on intact cells for the agonist zinterol was also the same regardless of whether incubations were carried out for 1 min or 45 min (Fig. 3; see ref. 15).

The effect of isoproterenol on the properties of receptors in membranes prepared from WT and *cyc*$^-$ cells was examined. Experiments with WT cells were carried out in the presence of 300 μM GTP to eliminate complexities due to the formation of a ternary complex between receptor, agonist, and the guanine nucleotide-binding protein (*G/F*) (25). The effect of isoproterenol on membranes prepared from *cyc*$^-$ cells was examined in the absence of GTP. Competition curves for isoproterenol in both types of membranes were shifted slightly (2- to 3-fold) to the right as a function of time (Fig. 6). At incubation times between 1 and 45 min, the competition isotherms best fit a single-affinity state of the receptor-agonist complex, and all of the specifically bound [^{125}I]IPIN was fully competed for by high concentrations of isoproterenol.

Competition experiments with isoproterenol carried out with intact WT cells showed a large time-dependent shift to the right (Fig. 7A). When the incubation time was increased from 1 to 45 min, the difference in apparent affinities was about 50-fold. Moreover, in contrast to results obtained with antagonists, the competition curves in WT cells were biphasic even under equilibrium conditions (45–60 min). Nonlinear regression analysis of these results revealed that, when short incubation times were used (0.5–1 min), about 80% of the receptor-agonist complex was in a high-affinity state. This fraction was reduced in a time-dependent fashion such that it stabilized at about 20% when assay times exceeded 30 min. After 30 min there was no further change in the shape of the curve or in the apparent affinity of the receptor for isoproterenol. An unexpected finding was that iso-

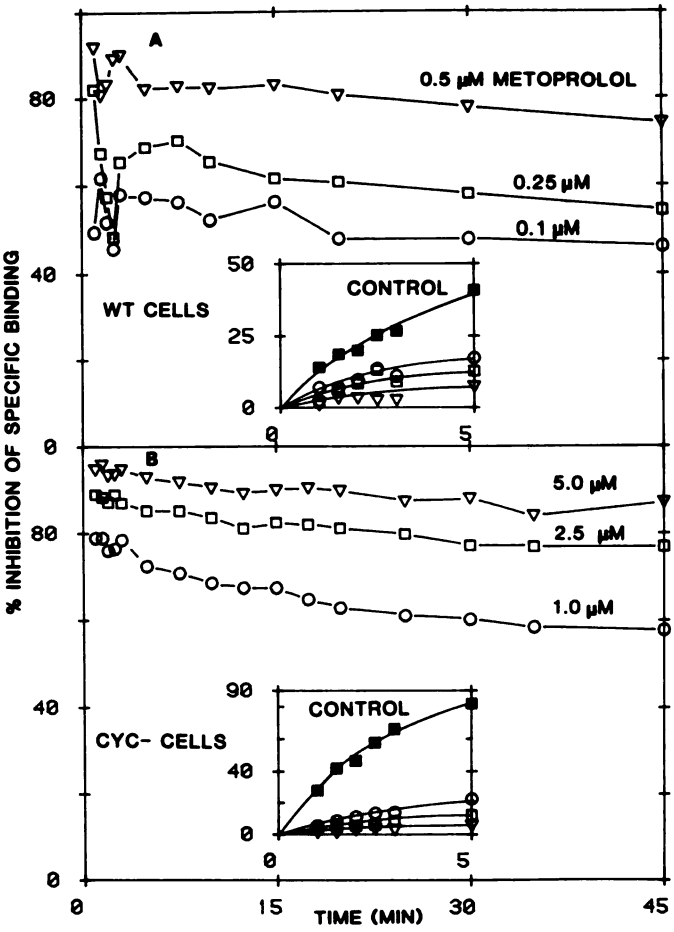

FIG. 4. *Time course of the binding of [^{125}I]IPIN to receptors on intact cells in the presence of metoprolol*

A. WT cells (400,000/250 μl) were incubated with [^{125}I]IPIN (95 pM) in the absence (■) or presence of 0.1 (○), 0.25 (□), or 0.5 (▽) μM metoprolol. B. *Cyc*$^-$ cells (300,000/250 μl) were incubated with [^{125}I]IPIN (100 pM) in the absence (■) or presence of 1.0 (○), 2.5 (□), or 5.0 (▽) μM metoprolol. The results are expressed as transformed data (see Fig. 1). The *insets* show the time course of the reaction for the first 5 min. Binding is expressed as percentage of specifically bound metoprolol at equilibrium in the control versus time (minutes). Total and nonspecific binding were determined in triplicate.

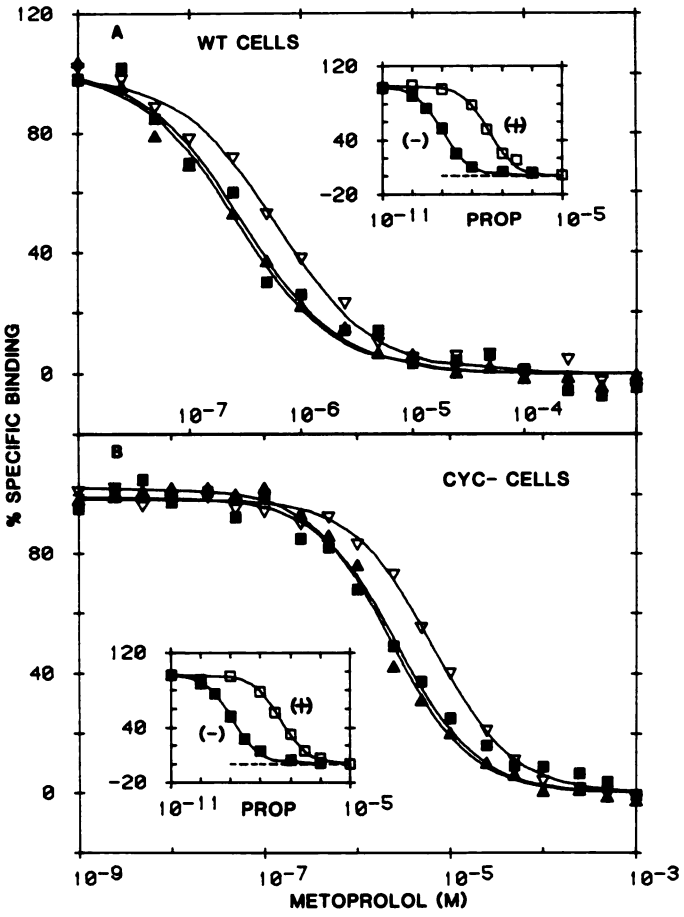

FIG. 5. *Inhibition of the binding of [^{125}I]IPIN by metoprolol under equilibrium and nonequilibrium conditions*

A. WT cells (300,000/250 μl) were incubated for different times with [^{125}I]IPIN (102 pM) and 16 concentrations of metoprolol. B. *Cyc*$^-$ cells (250,000/250 μl) were incubated with [^{125}I]IPIN (112 pM) and 20 concentrations of metoprolol. The results are expressed as percentage of specifically bound [^{125}I]IPIN versus the concentration of metoprolol at 1 min (■), 5 min (▲), or 45 min (▽). The *lines* represent the theoretical fit for a one-site model. Data points represent means from triplicate determinations. The data shown are representative of three and two experiments performed with WT and *cyc*$^-$ cells, respectively. *Insets.* A, WT cells; B, *cyc*$^-$ cells (500,000/assay) were incubated for 1 min with [^{125}I]IPIN (84 pM) and eight concentrations of (−)-propranolol (■) or (+)-propranolol (□) for 1 min. Points represent means of quadruplicate determinations.

proterenol was not able to inhibit all of the specifically bound [^{125}I]IPIN at short incubation times (0.5–2 min).

Competition experiments with isoproterenol were also carried out with *cyc*$^-$ cells (Fig. 7B). The competition curves were shifted to the right by a factor of between 50 and 100 when assays were carried out for 1 min as compared with assays carried out under equilibrium conditions. However, all of the complex was in a low-affinity state at equilibrium. As observed with WT cells, isoproterenol was not able to inhibit all of the specifically bound [^{125}I]IPIN when assays were carried out for very short times.

Similar results were obtained in studies carried out with the agonist Cc25 (data not shown). As with isoproterenol, most of the receptor-agonist complex was in a high-affinity state at short incubation times. In an additional set of experiments, isoproterenol competition experiments were performed at different incubation times with *kin*$^-$ and *unc* cells (data not shown). The results were similar to those obtained in WT cells.

Demonstration of sequestered receptors. Experiments were carried out to determine whether the 20% of the [^{125}I]IPIN binding sites not inhibited by isoproterenol at short incubation times were actually *beta*-adrenergic receptors. Competition experiments were performed with (−)- and (+)-propranolol in WT and *cyc*$^-$ cells in the presence of 10 μM isoproterenol. Assays were carried out using a 1-min incubation time. Under these conditions, isoproterenol did not fully compete for the [^{125}I]IPIN binding sites (Fig. 7). Although relatively little [^{125}I]IPIN was bound, the binding was fully stereoselective (Fig. 8),

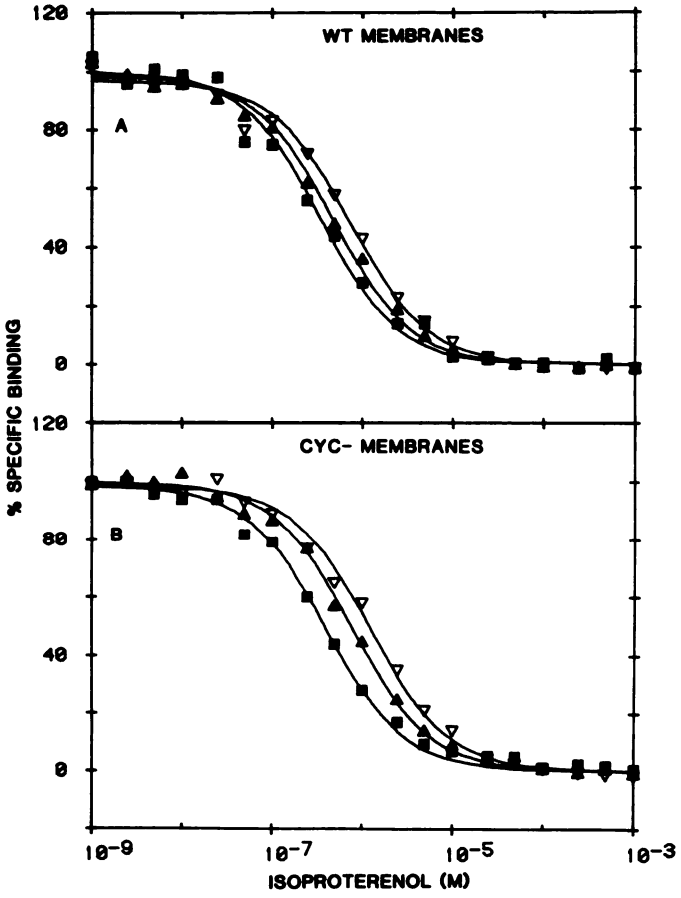

FIG. 6. *Inhibition of the binding of [^{125}I]IPIN to receptors on membranes by isoproterenol under equilibrium and non-equilibrium conditions*

A. Membranes from WT cells (3–5 μg/250 μl) were incubated for different times with [^{125}I]IPIN (89 pM) and 20 concentrations of isoproterenol in the presence of 300 μM GTP. B. Membranes from *cyc*$^-$ cells (3–5 μg/250 μl) were incubated with [^{125}I]IPIN (82 pM) and 20 concentrations of isoproterenol in the absence of GTP. The data best fit a single-state model. Results are expressed as percentage of specifically bound [^{125}I]IPIN versus the concentration of isoproterenol. The data points, means of triplicate determinations, show results obtained in assays lasting 1 min (■), 5 min (▲), or 45 min (▽). The data are representative of seven and five experiments performed with membranes from WT and *cyc*$^-$ cells, respectively.

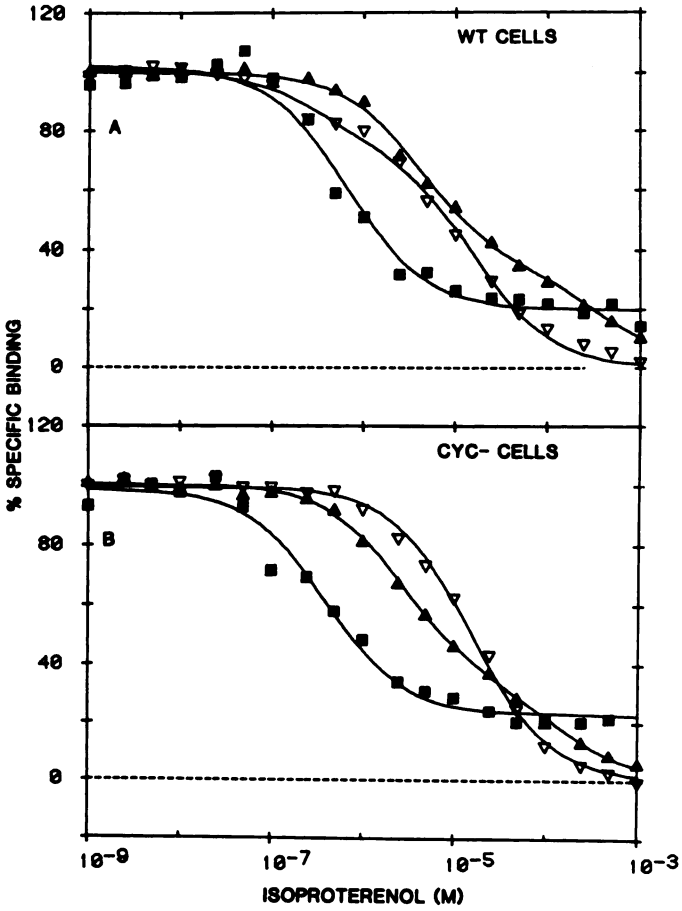

FIG. 7. *Inhibition of the binding of [^{125}I]IPIN to receptors on intact cells by isoproterenol under equilibrium and non-equilibrium conditions*

A. WT cells (600,000/250 μl) were incubated for different times with [^{125}I]IPIN (102 pM) and 20 concentrations of isoproterenol. B. *Cyc*$^-$ cells (400,000/250 μl) were incubated with [^{125}I]IPIN (109 pM) and 20 concentrations of isoproterenol. The figure represents 1-min (■), 5-min (▲), or 45-min (▽) competition curves expressed as percentage of specifically bound [^{125}I]IPIN versus the concentration of isoproterenol. The *lines* are computer-fit to the data points as described under Methods. The data points, means of triplicate determinations, are representative of 20 and 4 similar experiments with WT and *cyc*$^-$ cells, respectively.

indicating that this fraction of the binding is related to *beta*-adrenergic receptors. This population of sequestered receptors could be detected only at incubation times of less than 2 min. In competition experiments carried out for 5 min, isoproterenol was able to fully inhibit the binding of [^{125}I]IPIN (Fig. 7).

The lipophilic antagonists metoprolol and propranolol, in contrast to the hydrophilic agonists isoproterenol, epinephrine, and Cc25, were shown to compete for all of the specific binding sites for [^{125}I]IPIN even under non-equilibrium conditions. Competition experiments were also performed using sotalol, a hydrophilic antagonist. When the incubation time was 1 min, approximately 20% of the specific binding of [^{125}I]IPIN was not inhibited by sotalol in WT or *cyc*$^-$ cells (Fig. 9). This compound inhibited all of the specific binding of [^{125}I]IPIN under equilibrium conditions (*inset*, Fig. 9). The fraction of the binding of [^{125}I]IPIN not inhibited by sotalol was

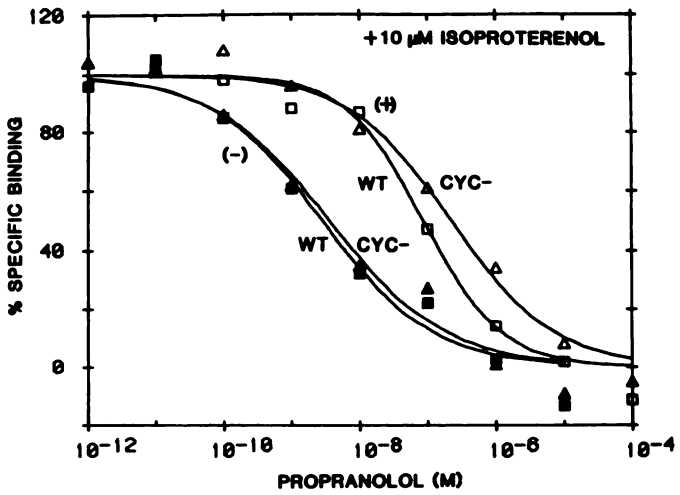

FIG. 8. *Stereospecificity of non-displaceable binding*

WT (■, □) and *cyc*$^-$ (▲, △) cells (500,000 and 570,000/250 μl) were incubated with [^{125}I]IPIN (173 pM), isoproterenol (10 μM), and nine concentrations of (−)-propranolol (■, ▲) or (+)-propranolol (□, △) for 1 min. The data are expressed as percentage of specifically bound [^{125}I]IPIN versus the concentration of propranolol. The *lines* represent the computer fit for one affinity state. Data points are means from triplicate determinations.

the same as was observed when experiments were carried out with isoproterenol (Fig. 7A and B). In contrast to the results observed in studies with hydrophilic agonists, the time-dependent decrease of the affinity of the receptors for sotalol was only 3- to 4-fold, similar to that observed for the lipophilic antagonists metoprolol and propranolol.

Other experiments were carried out with salbutamol, a lipophilic agonist. Competition curves were shifted to

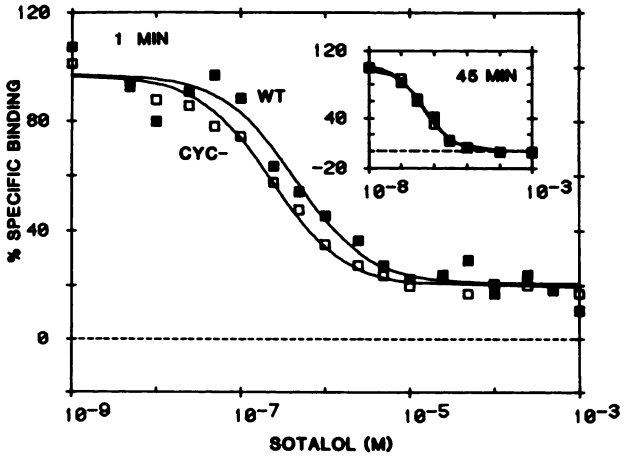

FIG. 9. *Inhibition of the binding of [^{125}I]IPIN by sotalol under equilibrium and non-equilibrium conditions*

WT (■) and *cyc*$^-$ (□) cells (400,000 and 750,000/250 μl) were incubated with [^{125}I]IPIN (84–108 pM) for 1 or 45 min (*inset*) in the presence of 19 concentrations of sotalol. The data are expressed as percentage of specifically bound [^{125}I]IPIN versus the concentration of sotalol. The *lines* were computer-fit as described. The data points are means of triplicate or quadruplicate determinations and are representative of three similar experiments.

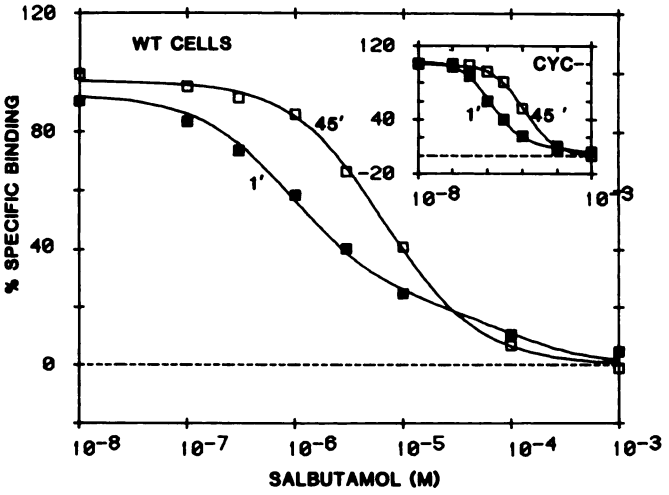

FIG. 10. *Inhibition of the binding of [^{125}I]IPIN by salbutamol under equilibrium and non-equilibrium conditions*

WT cells and *cyc*$^-$ cells (*inset*) (500,000/assay) were incubated for 1 (■) or 45 (□) min with [^{125}I]IPIN (84 pM) and eight concentrations of salbutamol. Points represent means of quadruplicate determinations. The data are representative of two and one experiments in WT and *cyc*$^-$ cells, respectively.

the right when assays were carried out for 45 min as compared with assays carried out under non-equilibrium conditions (Fig. 10). Unlike results observed with the hydrophilic compounds isoproterenol and sotalol, the binding of [^{125}I]IPIN was fully inhibited by salbutamol regardless of the duration of the assay. Similar results were observed in studies with *cyc*$^-$ cells (*inset*, Fig. 10).

DISCUSSION

Studies of the binding of labeled antagonists to *beta*-adrenergic receptors on intact cells have been reported in several systems (3–16). A common feature of most of these studies (6–10, 15, 16) was that the affinities of receptors for agonists were 2–3 orders of magnitude lower when binding studies were carried out with intact cells under equilibrium conditions as compared with studies with membrane preparations. The interactions of antagonists, on the other hand, were the same in cells and in membranes. Some authors have invoked spare receptors to explain these very low affinities (6, 7) and/or the presence of endogenous guanine nucleotides in intact cells (8). However, when studies were carried out with membranes in the presence of maximally effective concentrations of guanine nucleotides, affinities of receptors for agonists were frequently 1–2 orders of magnitude higher than those observed in studies with intact cells (10).

Pittman and Molinoff (10) showed that the affinity of receptors on intact L6 muscle cells for agonists was initially high but that it decreased within the first few minutes of incubation. This phenomenon has been investigated in detail in intact S49 lymphoma cells because mutants (*cyc*$^-$) have been isolated that appear to lack a functional *G/F* protein (26–28).

[^{125}I]IPIN was used in these experiments for three reasons: (a) nonspecific binding is lower than with [^{125}I] IHYP; (b) the active isomer of pindolol is available; and (c) the binding of [^{125}I]IPIN reaches equilibrium more rapidly than does the binding of [^{125}I]IHYP. These characteristics of the binding of [^{125}I]IPIN made it possible to perform binding studies under both equilibrium and non-equilibrium conditions. The binding of [^{125}I]IPIN to *beta*-adrenergic receptors on WT and *cyc*$^-$ S49 cells was rapid, fully reversible, saturable, of high affinity, stable over 90 min, and inhibited stereoselectively. Nonspecific binding was less than 5% of total binding when assays were carried out at the K_D value in intact cells and about 12–14% of total binding at 5 times the K_D value. Similar results were obtained with iodocyanopindolol (data not shown). These ligands, although lipophilic, are, nonetheless, useful ligands with which to study *beta*-adrenergic receptors on intact cells. Since nonspecific binding is very low, it appears that there is no substantial uptake of these ligands; therefore, extended washing procedures were not needed.

When competition experiments with agonists were performed under equilibrium conditions in intact cells and the results compared with those obtained in similar experiments with membranes, it was observed that the affinity of the receptors for agonists was much lower in intact cells than in membranes. This was true for both WT and *cyc*$^-$ cells. The same kinds of experiments carried out with antagonists revealed no significant differences between the properties of receptors on cells and membranes. The kinetics of this phenomenon were investigated. Small decreases in the apparent affinity of the receptor for antagonists were observed in studies with both cells and membranes when assays were carried out under equilibrium as compared with non-equilibrium conditions. For example, metoprolol displaced [^{125}I]IPIN more easily at short times than at equilibrium. This was also the case for isoproterenol when the interactions of this agonist were studied with receptors on membranes. This is probably due to the fact that the binding of these ligands reaches equilibrium in less time than does the binding of [^{125}I]IPIN. This results in an overestimate of the affinity of receptors for the competing ligand at short times.

The main difference between antagonists and agonists relates to the rapid and marked decrease in the ability of agonists to inhibit the binding of [^{125}I]IPIN to receptors on intact cells. This effect, seen in studies with most agonists, although not in studies with zinterol or with antagonists, was observed in both time-course and competition experiments, and occurred with a half-time of about 2–3 min. The rate of appearance and the magnitude of this effect were similar in WT and *cyc*$^-$ cells. It should be noted, however, that it is difficult to determine the time course of the decrease in the affinity of the receptor for agonists. Since the change in the properties of the receptor occurs quickly, the affinity of the receptor is likely to be underestimated even with very short incubation times. It is interesting to note that the time course of this effect was very similar to the time course of agonist-induced desensitization of adenylate cyclase in S49 cells as reported by Green *et al.* (29). The relationship of these two phenomena is currently under investigation.

When competition experiments were carried out with

isoproterenol in intact WT cells under equilibrium conditions (45–60 min), 80% of the receptor-agonist complex was in a low-affinity state, 20% being in a high-affinity state. When short incubation times were used (0.5–1 min), the receptor-agonist complex was exclusively in a high-affinity state. In *cyc*$^-$, *kin*$^-$, and *unc* cells, competition experiments also revealed a high-affinity state at short incubation times, but at equilibrium the receptor-agonist complex was exclusively in a low-affinity state. When competition experiments with isoproterenol were carried out for 2–30 min, the data analysis was more complex. The competition curves were biphasic and best fit a model with two affinity states.

A small but consistent fraction of the receptors appeared to be sequestered. In both WT and *cyc*$^-$ cells, approximately 20% of the specific binding of [^{125}I]IPIN was not inhibited by agonists or the hydrophilic antagonist sotalol at short incubation times. Binding to these sites was inhibited stereoselectively by propranolol and the lipophilic agonist salbutamol. Studies with salbutamol revealed a time-dependent decrease in the affinity of the receptor for this agonist, although specific binding of [^{125}I]IPIN was fully inhibited at 1 min. Sequestered receptors can be detected only at incubation times of less than 5 min, probably because high concentrations of hydrophilic ligands can equilibrate across the plasma membrane after several minutes. It is also possible that the sequestered receptors exist in equilibrium with receptors on the plasma membrane.

The agonist-induced changes in the properties of the receptor may be explained in several ways. One possibility is that agonists induce a specific change in the conformation of the receptor, such that receptors with a high affinity for agonists are converted to receptors with a low affinity for agonists. This explanation was previously suggested by Pittman and Molinoff (10, 15) to explain the low affinity of the receptor for agonists as measured at equilibrium. In our previous studies with L6 muscle cells, we identified some full agonists such as zinterol and salmefamol which did not cause any of the receptors to convert from a form with a high affinity for agonists to one with a low affinity for agonists. Other agonists, such as isoproterenol, caused all the receptors to convert from a form with a high affinity for agonists to a form with a low affinity for agonists. Similar findings were obtained in the present investigation in studies with zinterol and S49 lymphoma cells. The fact that zinterol does not cause formation of receptors with a low affinity for agonists in either L6 or S49 cells suggests that this finding is not an artifact of the use of L6 cells. Zinterol induces a marked increase in cyclic AMP on WT cells without inducing a change in the properties of the receptors. On the other hand, incubation of *cyc*$^-$ cells with isoproterenol led to a decrease in the affinity of the receptor for isoproterenol without inducing an increase in cyclic AMP levels. These results suggest that this is an agonist-specific effect that does not require increased production of cyclic AMP.

Several authors have explained high- and low-affinity states of the receptor for agonists by reference to the formation of a ternary complex (see ref. 25). According to this hypothesis, the ternary complex, *R-G/F-H*, represents the high-affinity state, whereas the binary complex of *R-H* represents the low-affinity state. Since similar results were obtained with WT and *cyc*$^-$ cells, it appears that a functional *G/F* protein is not required to explain the observed agonist-induced decrease in the affinity of the receptor for agonists. However, although *cyc*$^-$ cells are thought to be missing a functional stimulatory *G/F* protein (the 35K/45K dimer, or N_S), the 35K subunit of *G/F* protein is present in *cyc*$^-$ cells (30). Thus, although it is apparent that the functional stimulatory *G/F* protein (35K/45K) is not required for these changes in the affinity of the receptor for agonists, it is not possible to rule out the involvement of a ternary complex of *R*-35K-*H*.

Another explanation for the agonist-induced decrease in the affinity of the receptor for agonists can be developed based on the observation that some of the receptors are sequestered (see Fig. 7) (31, 32). This explanation would lead to the prediction that the apparent low-affinity sites would be observed at short incubation times but would disappear when an agonist equilibrates across the plasma membrane. However, from 80% to 100% of the receptors had a low affinity for agonists when measured at equilibrium. Furthermore, in competition experiments with the lipophilic agonist salbutamol, a time-dependent decrease in the affinity of the receptor for salbutamol was observed. Thus, it is unlikely that the sequestration of receptors can fully explain the low-affinity interactions that were observed in the current experiments.

Although it is not possible to rule out either the formation of a ternary complex or sequestration as playing a role in the agonist-induced decrease in the affinity of the receptor for agonists, the available data support the idea that it is primarily due to an agonist-induced change in the conformation of the receptor. Since this effect was not seen with the agonist zinterol, it is not a required step in the sequence of events leading from agonist occupancy of receptors to activation of adenylate cyclase. It has recently been reported that desensitization of *beta*-adrenergic receptors in turkey erythrocytes is related to phosphorylation of the *beta*-adrenergic receptor (33). A change of this type could account for the change in conformation associated with a decrease in the affinity of the receptor for agonists. In any event, the results reported underline the usefulness of kinetic studies and non-equilibrium binding experiments with intact cells. Furthermore, the demonstration of sequestered receptors in intact cells may have important implications for the functional status of *beta*-adrenergic receptor-adenylate cyclase coupling. The possibility that the properties or the proportion of sequestered receptors may be modulated by agonists is presently under investigation.

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