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High-affinity binding of agonists to β-adrenergic receptors on intact cells

(nonequilibrium binding assays/cyclic AMP formation/desensitization)

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ABSTRACT **The interactions of agonists and antagonists with β-adrenergic receptors on intact 1321N1 human astrocytoma and C62B rat glioma cells were studied by using the radioligand (−)-[^{125}I]iodopindolol. Competition binding assays were performed at 37°C under equilibrium conditions and in short-time nonequilibrium assays that approximated initial velocity conditions for binding of the radioligand. The theoretical basis and experimental validation of the initial velocity approach for determining binding affinities of rapidly equilibrating ligands are presented. For the agonists isoproterenol and epinephrine, high binding affinities that approximated their apparent affinities for binding in membranes and for increase of cyclic AMP concentrations in intact cells could be demonstrated only in short-time assays; in contrast, much lower affinities were observed with equilibrium (60-min) assays as reported previously for various cell lines. High-affinity binding of isoproterenol to 1321N1 cells also was observed in equilibrium (6-hr) binding assays carried out on ice. These results indicate that in the native state the intact cell β-adrenergic receptor has a high binding affinity for agonists and suggest that incubation at 37°C in the presence of an agonist converts the receptors to a form with low affinity for agonists.**

The apparent binding affinity of β-adrenergic receptor *agonists*, as measured by their capacity to inhibit radiolabeled antagonist binding to intact cells, has been shown to be markedly lower than the apparent affinity of these agonists in stimulation of cyclic AMP accumulation and in binding to receptors in membrane preparations (1–3). In contrast, the binding affinities for *antagonists* are in good agreement both with their apparent affinities in inhibition of agonist-stimulated cyclic AMP accumulation in intact cells and with their affinities in binding to receptors in membrane preparations. In assays of membrane preparations, receptor binding affinities for both agonists and antagonists are in good agreement with their apparent affinities in stimulation or inhibition of adenylate cyclase.

Although the reason for the low affinity of agonists observed in intact cell binding assays is unknown, it is well recognized that exposure of cells to agonists can lead to rapid changes in both the physical and pharmacological properties of β-adrenergic receptors (4–9). Thus, agonist-induced conversion of β-adrenergic receptors from a native form with high affinity for agonists to a low-affinity form during the time course of an equilibrium competition binding assay could account for these results. Two previous studies have considered this possibility. Terasaki and Brooker (1) were unable to detect high-affinity binding of agonists to C62B rat glioma cells at assay times as short as 2.5 min, and they suggested that the low binding affinity observed was due to the existence of "spare receptors." They concluded that binding to as little as one receptor per C62B cell was sufficient to fully activate adenylate cyclase. In contrast, Pittman and Molinoff (2), using a different cell line (L6 rat muscle cells), demonstrated that isoproterenol inhibited radiolabeled antagonist binding at short assay times (<2 min) more effectively than at longer incubation times and presented evidence suggesting that the low binding affinity observed in equilibrium assays was due to a rapid change in receptor affinity occurring during incubation of these cells with agonists.

To test the possibility that rapid decreases in binding affinity for agonists might be occurring during the time course of equilibrium binding assays, an approach was developed for the determination of equilibrium dissociation constants (K_d) for competing ligands by using short-time competition binding assays under nonequilibrium conditions of binding of the radioligand. By using this method, high-affinity binding of agonists to β-adrenergic receptors of intact 1321N1 human astrocytoma cells and C62B rat glioma cells can be readily demonstrated. High-affinity binding of agonists also is expressed even after prolonged incubation in the presence of agonist to achieve equilibrium binding if the assays are performed at reduced temperature.

MATERIALS AND METHODS

Materials. (−)-Isoproterenol bitartrate and (±)-propranolol hydrochloride were obtained from Sigma. The following drugs were generous gifts: (−)-pindolol from Sandoz Pharmaceutical, (−)-epinephrine bitartrate from Sterling-Winthrop Research Institute (Rensselaer, NY), (−)-propranolol hydrochloride from Ayerst Laboratories (New York), and (±)-metoprolol from P. B. Molinoff and B. B. Wolfe. C62B rat glioma cells were generously provided by Gary Brooker and Ken Barovsky. (−)-[^{125}I]-Iodopindolol (^{125}I-Pin) was prepared by a modification (10) of the method of Barovsky and Brooker (11).

Cell Culture. Human astrocytoma cells (1321N1) were maintained in Dulbecco's modification of Eagle's medium supplemented with 5% fetal calf serum. Rat glioma cells (C62B, passage 30–40) were maintained in Eagle's medium supplemented with pyruvic acid (1 mM), asparagine (0.2 mM), serine (0.2 mM), and 5% fetal calf serum. Growth of cells was carried out under an atmosphere of 92% air and 8% CO_2 at 37°C in a humidified incubator. Cells taken from confluent flasks were seeded in 35-mm culture dishes (Falcon) at a density of 80,000 cells per dish and used on the fourth day after subculture.

Binding of ^{125}I-Pin to Intact Cells. The binding assay used for 1321N1 cells was a modification of the procedure of Barovsky and Brooker (11). Cell sheets were rinsed with 2 ml of serum-free Eagle's minimal essential medium containing 20 mM Hepes (pH 7.4). One milliliter of Eagle's/Hepes medium containing ^{125}I-Pin, 1 mM sodium ascorbate, and various concentrations

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Abbreviation: ^{125}I-Pin, (−)-[^{125}I]iodopindolol.

of competing ligand was added to each dish and the cells were incubated at 37°C for the time indicated. The binding medium was then aspirated and the cells were given two 1-min washes at 37°C (1 ml each) with Eagle's/Hepes medium containing (±)-propranolol (100 μM). Cell-associated radioactivity was determined after solubilization of the monolayer with 0.2 M NaOH. Nonspecifically bound radioligand [that bound in the presence of 1 μM (±)-propranolol] dissociated rapidly at 37°C and was about 5% (equilibrium assays) or 20% (short-time assays) of total binding after 2 min of washing. Specifically bound radioligand dissociated slowly, with only about 3% lost during the 2-min wash. Both kinetic and equilibrium binding studies were consistent with binding of ^{125}I-Pin to a single class of sites with an equilibrium K_d of approximately 10 pM.

Binding of ^{125}I-Pin to β-adrenergic receptors on 1321N1 cells also was measured at a lower temperature (on ice), using the same wash procedure as for the 37°C assays. Equilibrium binding was obtained after 6 hr, and Scatchard analysis indicated the same number of receptors labeled on ice as were labeled at 37°C. The K_d value for ^{125}I-Pin binding on ice was approximately 25 pM.

The binding assay used for C62B cells was the same as that for 1321N1 cells except that the wash procedure was modified due to the rapid dissociation of specifically bound radioligand at 37°C. C62B cells were given a 1-min wash followed by a 9-min wash (1 ml each) on ice. Less than 5% of specific binding was lost during this wash procedure; nonspecific binding was approximately 15% (equilibrium assays) or 50% (short-time assays) of total binding present after washing. The K_d for ^{125}I-Pin binding to C62B cells at 37°C was approximately 100 pM.

Data Analysis. Competition curves were analyzed by computerized nonlinear least-squares curve fitting of the raw data by using the Gauss–Newton method (12). The model used was that for law of mass action interaction of the competing ligand with either a single site (n = 1) or two independent sites (n = 2) as follows:

$$B_S = \sum_{i=1}^{n} B_i - \sum_{i=1}^{n} \frac{B_i S}{I_i + S} + N, \qquad [1]$$

in which S is the concentration of competing ligand, B_S is the concentration of ^{125}I-Pin bound at a given value of S, B_i is the concentration of specific binding sites, I_i is the IC_{50} for the competing ligand at each binding site, and N is the concentration of nonspecific binding sites. It should be noted that this model is appropriate only if the competing ligand is at equilibrium with all specific binding sites under the conditions of the assay.

The single-site and two-site models were compared as described (13). Values reported for fractions of high- and low-affinity binding sites are reported as arithmetic means; values for IC_{50} and K_d are reported as geometric means (14). In all figures, the data are expressed as a percentage of ^{125}I-Pin specifically bound in the absence of competing drug. Specific binding in the absence of competing drugs in 1321N1 cells was approximately 1,500 cpm in short-time assays and approximately 9,000 or 16,000 cpm in equilibrium assays at 25 pM or 165 pM ^{125}I-Pin, respectively. The values for C62B cells were approximately 500 cpm bound per assay at 15 sec and 5,000 cpm bound per assay at 20 min.

THEORETICAL CONSIDERATIONS

If receptor binding of a competing ligand attains equilibrium much more rapidly than does that of the radioligand, the concentration of free receptors at early times of incubation will be essentially

$$R_f = \frac{R_t}{1 + [C]/K_{d1}}, \qquad [2]$$

in which R_f is free receptor concentration, R_t is total receptor concentration, $[C]$ is the concentration of free competitor, and K_{d1} is the equilibrium dissociation constant for the competing ligand. If the concentration of receptor-bound radioligand ($[LR]$) is measured at a sufficiently early time such that initial velocity assumptions apply ($[LR] \ll [LR]_{eq}$), then $[LR]$ can be approximated as

$$[LR] = k_{on} t [L] R_f, \qquad [3]$$

in which k_{on} is the association rate constant for the radioligand, t is the time of binding, and $[L]$ is the concentration of free radioligand. Substituting the expression for R_f from Eq. 2 yields

$$[LR] = \frac{k_{on} t [L] R_t}{1 + [C]/K_{d1}}. \qquad [4]$$

The IC_{50} is defined as the concentration of competing ligand required to reduce the amount of radioligand specifically bound to half the value obtained in the absence of competitor. From Eq. 4 it can be seen that this will occur when $[C] = K_{d1}$. Thus, the IC_{50} obtained in binding assays under initial velocity conditions provides an estimate of the equilibrium dissociation constant for competing ligands that come to equilibrium very rapidly. As the time at which binding is measured increases, the competition curves will shift progressively further to the right until equilibrium is attained, at which time the binding can be described by the following equation:

$$[LR] = \frac{R_t}{1 + (K_{d2}/[L])(1 + [C]/K_{d1})}, \qquad [5]$$

in which K_{d2} is the equilibrium dissociation constant for the radioligand. The extent to which the IC_{50} of the equilibrium curve is shifted rightward from the true K_d is determined by the concentration of radioligand used. The true K_d can be obtained by dividing the IC_{50} by the factor $(1 + [L]/K_{d2})$ (15).

It should be stressed that this initial velocity method for K_d determination will be valid only if the competing ligand comes to equilibrium very rapidly relative to the time over which binding is measured. If the competitor comes to equilibrium slowly relative to the time of the assay, the competition curves obtained will appear to the right of the true K_d due to incomplete equilibration of the competitor (16, 17).

RESULTS

Competition Binding Assays with Antagonists. The validity of the initial velocity method for estimating K_d values was tested by generating competition binding curves for *antagonists* with intact 1321N1 cells under both initial velocity and equilibrium conditions. The β-adrenergic receptor antagonists metoprolol (Fig. 1) and propranolol (Fig. 2) both inhibited binding of ^{125}I-Pin with properties consistent with law of mass action interaction with a single class of sites. The K_d values calculated from equilibrium (60-min) assays with metoprolol (K_d = 240 ± 5 nM, n = 4) and propranolol (K_d = 99 ± 8 pM, n = 3) (all values are given ± SEM) were both in good agreement with K_d values obtained in binding assays with membrane preparations and with the K_i values of the two antagonists for inhibition of isoproterenol-stimulated cyclic AMP accumulation in intact cells.

For the rapidly equilibrating antagonist metoprolol, the IC_{50} (320 ± 13 nM, n = 4) obtained in 1-min assays (Fig. 1) or 15-

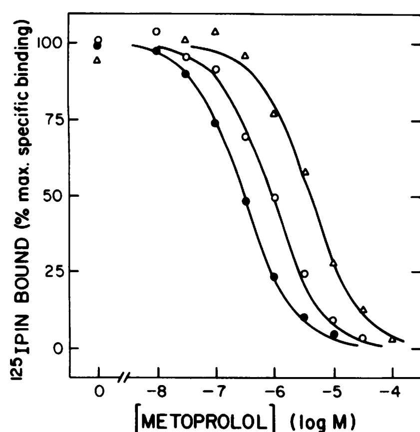

FIG. 1. Competition binding curves for metoprolol with intact 1321N1 cells. ^{125}I-Pin binding to 1321N1 cells was determined in the presence of the indicated concentrations of (±)-metoprolol at 37°C in either 1-min (●) or 60-min (○, △) assays. The concentration of ^{125}I-Pin used was either 25 pM (●, ○) or 165 pM (△). The points shown are the averages of two experiments.

sec assays (data not shown) was in good agreement with the K_d calculated from equilibrium assays. The shift observed between the initial velocity curve and the equilibrium curves obtained at two different radioligand concentrations (Fig. 1) was thus very near that predicted by the Cheng–Prusoff equation (15).

Propranolol is a higher affinity ligand than metoprolol and attains equilibrium only slowly at concentrations near its K_d. The competition curve obtained in 1-min assays with propranolol (Fig. 2, IC_{50} = 1,600 pM) was shifted to the right relative not only to the theoretical position (broken line, K_d = 99 pM) but also to the position of the curve in equilibrium assays. The curve obtained in 15-sec assays was shifted even further to the right (data not shown). This is the result expected for a slowly equilibrating competitor (16, 17). When cells were preincubated for 60 min with various concentrations of propranolol to allow equilibration with the receptor prior to 1-min competition assays, an experimental curve indistinguishable from the theoretical curve (Fig. 2, broken line) was obtained. Thus, the 1-min assay curve in Fig. 2 is shifted to the right due to incomplete equilibration of the competing ligand and is not an artifact due to nonequilibrium of ^{125}I-Pin with the receptor.

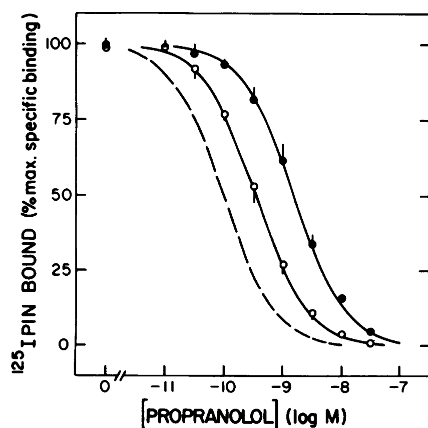

FIG. 2. Competition binding curves for propranolol with intact 1321N1 cells. 1321N1 cells were incubated with ^{125}I-Pin (25 pM) and the indicated concentrations of (−)-propranolol at 37°C for either 1 min (●) or 60 min (○). The results are the mean ± SEM of three experiments. The broken line is the theoretical binding curve for a competing ligand with K_d = 99 pM, which is the value obtained for propranolol in equilibrium assays with intact cells.

The results from these studies of *antagonist* competition for ^{125}I-Pin binding indicate that short-time assays can provide a valid measure of the equilibrium dissociation constant for rapidly equilibrating competing ligands; for competing ligands that do not attain equilibrium sufficiently rapidly, short-time assays will provide an overestimate of the true K_d value.

Competition Binding Assays with Agonists. *Agonist* competition curves obtained in equilibrium (60-min) binding assays with 1321N1 cells (Fig. 3) were significantly ($P < 0.05$) better fit by a two-component model than by a model for law of mass action interaction with a single site. For isoproterenol, 13 ± 2% (n = 4) of ^{125}I-Pin binding was inhibited with an IC_{50} of 5.9 ± 1.3 nM and the remaining 86% was inhibited with an IC_{50} of 41 ± 2 μM. For epinephrine, 17 ± 4% (n = 3) of ^{125}I-Pin binding was inhibited with an IC_{50} of 54 ± 8 nM and the remaining 83% was inhibited with an IC_{50} of 870 ± 52 μM. Previous studies (7, 18) have shown that incubation of these cells in the presence of agonists for times as short as 60 min induces measurable receptor loss. The apparent inhibition of binding seen at low concentrations of these agonists more likely represents a concentration-effect curve for this receptor loss phenomenon than inhibition by the agonists of ^{125}I-Pin binding to high-affinity receptors still present on the cells. The K_d values (11 ± 1 μM for isoproterenol, 240 ± 12 μM for epinephrine) calculated from the lower-affinity IC_{50} values are much higher than the K_{act} values (15 nM for isoproterenol, 50 nM for epinephrine) for stimulation of cyclic AMP accumulation in intact cells and are also much higher than the K_d values (38 nM for isoproterenol, 210 nM for epinephrine) for inhibition of binding to receptors in membrane preparations. These results are similar to those previously reported in studies of other cultured cells (1–3); it is this inconsonance between K_d and K_{act} values that our studies seek to explain.

Inhibition of ^{125}I-Pin binding to cells by isoproterenol and epinephrine also was measured at short times of incubation.

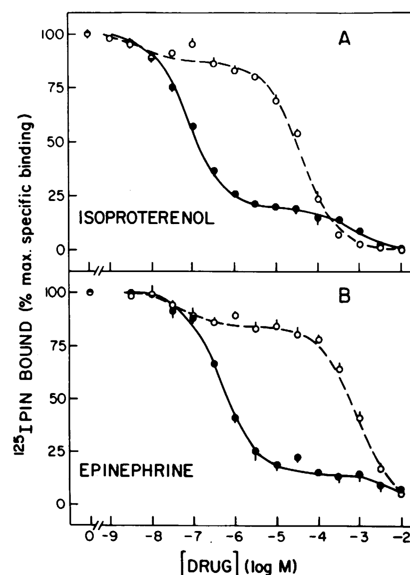

FIG. 3. Agonist competition binding curves with intact 1321N1 cells. Intact 1321N1 cells were incubated with ^{125}I-Pin and the indicated concentrations of (−)-isoproterenol (*A*) or (−)-epinephrine (*B*) at 37°C for either 15 sec (●) or 60 min (○). The concentration of ^{125}I-Pin used was 25 pM in the 60-min assays and between 50 and 150 pM in different experiments involving 15-sec assays. Each data point is the mean ± SEM of two to five experiments for isoproterenol and of two or three experiments for epinephrine.

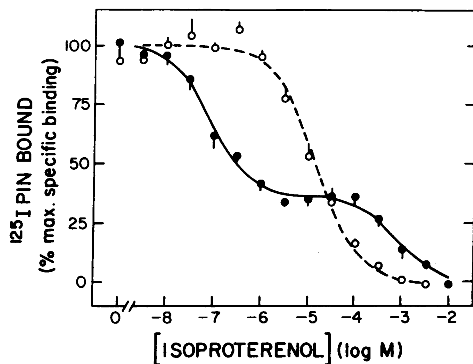

FIG. 4. Competition binding curves for isoproterenol with intact C62B cells. C62B cells were incubated with ^{125}I-Pin and the indicated concentrations of (−)-isoproterenol at 37°C for either 15 sec (●) or 20 min (○). The concentration of ^{125}I-Pin used in different experiments was between 25 and 100 pM for 15-sec assays and between 10 and 25 pM for 20-min assays. The results are the mean ± SEM from three experiments.

When 15-sec assays were performed (Fig. 3), these agonists were much more potent as inhibitors of ^{125}I-Pin binding than was observed after a 60-min equilibrium binding assay. These data also were better fit by a two-component model. For isoproterenol, 81 ± 1% (n = 5) of ^{125}I-Pin binding was inhibited with an IC_{50} of 81 ± 4 nM and the remaining 19% was inhibited with an IC_{50} of 660 ± 130 μM. For epinephrine, 85 ± 4% (n = 2) of ^{125}I-Pin binding was inhibited with an IC_{50} of 470 ± 5 nM and 15% was inhibited with an IC_{50} of 7,600 ± 760 μM.

Competition binding assays with isoproterenol also were performed with C62B rat glioma cells. The curve obtained in equilibrium assays (20 min, Fig. 4) was consistent with law of mass action interaction of isoproterenol with a single class of sites (K_d = 9.3 ± 0.7 μM, n = 3), in good agreement with previous results (1, 11). This value is much higher than the K_{act} for isoproterenol-stimulated cyclic AMP accumulation in these cells [10 nM (1)] and than the K_d for inhibition by isoproterenol of binding to receptors in membrane preparations [10–60 nM (1)]. The curve obtained in 15-sec assays (Fig. 4) was better fit by a two-component model, with 64 ± 2% (n = 3) of ^{125}I-Pin binding inhibited with an IC_{50} of 85 ± 12 nM, and 36% inhibited with an IC_{50} of 850 ± 34 μM. The results obtained in 15-sec assays clearly indicate that in both 1321N1 and C62B cells there is a high-affinity interaction of agonists with β-adrenergic receptors that is not detected in longer time equilibrium binding assays at 37°C.

Isoproterenol competition for ^{125}I-Pin binding to intact 1321N1 cells also was measured in equilibrium (6-hr) assays on ice with 100 pM ^{125}I-Pin (data not shown). The data were better fit by a two-component model with 86 ± 2% (n = 3) of ^{125}I-Pin binding inhibited with an IC_{50} of 270 ± 35 nM (K_d = 53 ± 6 nM) and the remaining 14% inhibited with an IC_{50} of 68 ± 28 μM.

DISCUSSION

A valid measure of the equilibrium dissociation constant for a competing ligand can be obtained under nonequilibrium conditions of radioligand binding if two criteria are satisfied: (*i*) the assay must approximate initial velocity conditions for radioligand binding, and (*ii*) the competing ligand must be at equilibrium with the receptor essentially throughout the time of radioligand binding (see *Theoretical Considerations*). The high affinity of ^{125}I-Pin for intact cell β-adrenergic receptors and the low level of nonspecific binding obtained even at short assay times have allowed competition binding assays to be carried out under conditions in which the radioligand–receptor interaction is approximately at the initial velocity; thus the amount of binding obtained in the absence of competitor in the short-time assays was about 10% of that obtained in equilibrium assays. With regard to the second criterion, previous studies suggest the rapid attainment of equilibrium binding of isoproterenol to β-adrenergic receptors on intact cells (19, 20). Rapid equilibration also might be expected for epinephrine and metoprolol, because their K_d values are similar to that for isoproterenol. The most important point with regard to these criteria, however, is that violation of either the initial velocity of radioligand binding assumption or the rapid equilibration of competitor assumption can lead only to an *over*estimate of the true value of the K_d for the competitor; thus the affinity of intact cell β-adrenergic receptors for these ligands must be at least as high as indicated by the short-time assays reported here. For the antagonist metoprolol (Fig. 1), the good agreement between the IC_{50} value obtained in short-time assays and the K_d value calculated from equilibrium binding assays suggests that both assumptions were valid. This was not the case for the antagonist propranolol (Fig. 2), which exhibits a $t_{1/2}$ to equilibrium of approximately 2 min at concentrations near its K_d (data not shown); the slow equilibration of propranolol resulted in IC_{50} values from short-time assays that were much larger than the true K_d.

Results obtained in short-time binding assays with isoproterenol and epinephrine in 1321N1 cells and with isoproterenol in C62B cells suggest that agonists interact with two distinct types of ^{125}I-Pin binding sites having markedly different agonist affinities. In all three cases the majority of the sites were of high affinity, with K_d values only 5- to 10-fold higher than their corresponding K_{act} values for stimulation of cyclic AMP accumulation (summarized in Table 1). It is most likely through interaction with these higher affinity sites that agonists act to stimulate cyclic AMP accumulation. These higher-affinity K_d values are also in good agreement with the K_d values determined in assays with membrane preparations. The nature and significance of the ^{125}I-Pin binding sites exhibiting the lower affinity for agonists in short-time assays remains to be determined.

The results obtained in equilibrium assays with these agonists in 1321N1 cells also appeared to indicate interaction with two types of ^{125}I-Pin binding sites of very different affinity. However, the apparent high-affinity interaction observed at low agonist concentrations most likely reflects agonist-induced loss of ^{125}I-Pin binding sites during the assay (7, 18). Thus after a 60-min incubation in the presence of agonists, only low-affinity interaction of agonists is observed. A single low-affinity interaction also was observed in equilibrium assays with C62B cells. The K_d values obtained in these equilibrium assays are 100- to 500-fold higher than those observed in the short-time assays and 500- to 5,000-fold higher than their corresponding K_{act} values for stimulation of cyclic AMP accumulation (Table 1).

Previous work (1) had suggested that the low agonist affinity observed in equilibrium assays with C62B cells was due to the existence of "spare receptors" and not due to agonist-induced

Table 1. Summary of K_d and K_{act} values

Cells	Ligand	K_d, nM Equilibrium	K_d, nM 15-sec assay	K_d, nM Membranes	K_{act} for intact cells, nM
1321N1	Isoproterenol	11,000	81	38	15
	Epinephrine	240,000	470	210	50
C62B	Isoproterenol	9,300	85	10–60	10

formation of a form of the receptor with low affinity for agonists as described for L6 cells (2). From experiments in which intact 1321N1 or C62B cells were incubated with agonists before short-time competition binding assays were performed, we have obtained evidence for a rapid and reversible agonist-induced conversion of intact cell β-adrenergic receptors from the high-affinity form detected in short-time assays to a form of lower affinity (unpublished data). This phenomenon most likely is reponsible for most of the discrepancy observed between K_{act} values and equilibrium K_d values. The remaining discrepancy may be due to the coupling relationships between the β-adrenergic receptor, the guanine-nucleotide binding protein, and the enzyme adenylate cyclase. Ratios of K_d/K_{act} that are somewhat greater than 1.0 are not unexpected for coupled systems involving several components (21–23).

A high-affinity interaction between isoproterenol and intact 1321N1 cell β-adrenergic receptors also was observed in equilibrium competition binding assays on ice. Insel and Sanda (24) reported similar results in studies of S49 mouse lymphoma cells, with agonists exhibiting higher binding affinity at 4°C than at 37°C. These results may indicate that binding interactions between receptor and ligands, both agonists and antagonists, can occur normally on ice, but that a subsequent reaction that results in formation of a low-affinity form of the receptor at 37°C is blocked at low temperatures.

The results presented here confirm and extend those of Pittman and Molinoff (2) and demonstrate that in a variety of cell lines the β-adrenergic receptor initially exhibits high-affinity agonist binding, but that agonist binding subsequently converts these receptors to a form exhibiting much lower agonist affinity. Further studies of agonist-induced changes in the properties of intact cell β-adrenergic receptors (unpublished) suggest that agonists promote internalization of cell surface receptors. The relationship of the agonist-induced change in agonist binding affinity observed with intact cells to receptor internalization and to the various aspects of catecholamine-induced desensitization of the β-adrenergic receptor-linked adenylate cyclase system that have been described previously (5, 7–9) remain to be investigated.

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