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Interactions of Full and Partial Agonists with *Beta*-Adrenergic Receptors on Intact L6 Muscle Cells

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SUMMARY

A nonfusing variant of L6 muscle cells was used to study the interactions of 16 agonists and 8 antagonists with *beta*-adrenergic receptors. Membranes prepared from L6 cells and intact cells in monolayer culture were used. *Beta*-adrenergic receptors on broken cells and on intact cells had the same affinities for all of the antagonists studied. Studies of the inhibition of the binding of [^{125}I]iodohydroxybenzylpindolol by agonists indicated that two states of the receptor can exist on intact cells attached to the substratum. The form of the receptor normally present on intact cells appeared to have the same properties as receptors on membranes when assayed in the presence of GTP. Several full agonists converted this form of the receptor to a form which had a 40- to 50-fold lower affinity for agonists. This conversion appeared to occur during the first few minutes of exposure to an agonist. Four of the agonists tested did not convert any of the receptors on intact cells to a form with a low affinity for agonists. Included in this group of agents were two full agonists and two partial agonists. Therefore, interactions of these drugs with receptors on broken or intact cells were the same. Several other full and partial agonists converted some of the receptors on intact cells to a low-affinity form, and their interactions with receptors on intact cells were characterized by shallow inhibition curves. The conversion of *beta*-adrenergic receptors on intact cells to a low-affinity state did not appear to be a prerequisite for the decrease in the rate of agonist-stimulated cyclic AMP accumulation that occurs 1–2 min after exposure of L6 cells to agonists. Studies were also carried out on viable intact cells detached from plates following brief exposure to trypsin or EDTA. The properties of receptors on suspended cells were the same as those of receptors on broken cells when assayed in the presence of GTP, rather than being similar to the properties of receptors on attached cells. In summary, data are presented indicating that agonists with the same potency and intrinsic activity in membrane preparations (and intact cells in suspension) can interact very differently with *beta*-adrenergic receptors on intact cells attached to the substratum. Thus, certain agonists cause a rapid conversion of *beta*-adrenergic receptors to a form which has a low affinity for agonists. This effect is seen with some but not all agonists and is seen only in studies with attached cells.

INTRODUCTION

Interactions of agonists and antagonists with *beta*-adrenergic receptors are markedly different. Agonists stimulate adenylate cyclase activity and ultimately induce one or more physiological responses. Antagonists, on the other hand, appear to occupy receptors without inducing a physiological response. Furthermore, experiments carried out in several laboratories have shown that divalent cations increase the affinity of *beta*-adrenergic receptors for agonists, whereas the affinity of receptors for agonists is decreased when assays are carried out in the presence of guanine nucleotides. Neither of these effects has been observed in studies of the interactions of antagonists with *beta*-adrenergic receptors (1–4). The energetics of the interactions of agonists and antagonists with *beta*-adrenergic receptors are also different (5, 6). Interactions of antagonists are entropy-driven, whereas the interactions of agonists are driven by decreases in enthalpy associated with thermodynamically unfavorable decreases in entropy. Finally, exposure of tissues or cells to agonists, but not antagonists, can initiate the process of desensitization in which there is a decrease in the ability of the system to respond to an appropriate challenge with an agonist (7–9).

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Recently, several attempts have been made to develop binding assays to study *beta*-adrenergic receptors on intact cells using clonal cell lines (10–13). It was reported (10, 11) that the concentrations of antagonists required to inhibit the binding of [^{125}I]IHYP[2] to receptors on intact cells were approximately the same as those required to inhibit isoproterenol-stimulated cyclic AMP accumulation. The agonist isoproterenol, on the other hand, stimulated cyclic AMP accumulation at concentrations 500 (11), 2000 (13), or 4000 (10) times lower than those required to inhibit radioligand binding in intact cells. These groups also reported that binding of antagonists appeared to be identical in intact cells and in membrane preparations; however, there were major differences in the interactions of agonists with *beta*-adrenergic receptors on broken and intact cells. Specifically, isoproterenol had a 3-fold (11) or 100-fold (10, 13) lower potency for inhibition of [^{125}I]IHYP binding in intact cells than in membrane preparations when assays were carried out in the presence of GTP. We have shown that, for L6 muscle cells, the discrepancy between the potency of isoproterenol for inhibition of [^{125}I]IHYP binding in membranes and intact cells is explained by a rapid, agonist-induced decrease in the affinity of receptors on intact cells for agonists (13). This alteration in the properties of the receptors was not observed following homogenization of cells or following exposure of cells to antagonists.

In the present study, we have used L6 muscle cells which have a high density (20,000 receptors/cell) of apparently homogeneous $beta_2$-adrenergic receptors (13, 14). A series of equilibrium binding experiments was carried out with the radioligand [^{125}I]IHYP and varying concentrations of 16 full and partial agonists. Experiments were also designed to determine whether the conversion of receptors on intact cells to a low-affinity form affected the rates of accumulation of cyclic AMP, and to determine whether the low-affinity form of the receptor was also formed on intact cells that had been detached and were in suspension.

MATERIALS AND METHODS

Drugs. The following drugs were used: Practolol and (+)- and (−)-propranolol (Ayerst Laboratories, New York, N. Y.); sotalol, soterenol, and zinterol (Mead Johnson and Company, Evansville, Ind.); phentolamine, terbutaline, and metoprolol (Ciba-Geigy Corporation, Summit, N. J.); (+)-isoproterenol (Sterling Winthrop Laboratories, Rensselaer, N. Y.); salmefamol and salbutamol (Allen and Hanburys, Toronto, Ont., Canada); fenoterol and metaproterenol (Boehringer Ingelheim, Ltd., Elmsford, N. Y.); pindolol and hydroxybenzylpindolol (Sandoz Pharmaceuticals, Hanover, N. J.); OPC 2009 (Hässle, Göteborg, Sweden); atenolol (ICI Americas, Inc., Stanford, Conn.); timolol (Merck, Sharp & Dohme, West Point, Pa.); carbuterol and sulfonterol (Smith Kline & French Laboratories, Philadelphia, Pa.); dobutamine (Eli Lilly and Company, Indianapolis, Ind.); Cc-25 (N. V. Phillips Duphar, Weesp, Holland).

Butoxamine was purchased from Burroughs-Wellcome (Research Triangle Park, N. C.). All other drugs and reagents used were commercially available.

[2] The abbreviations used are: [^{125}I]IHYP, [^{125}I]iodohydroxybenzylpindolol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMEM, Dulbecco's modification of Eagle's medium.

Cell culture. A nonfusing variant of the L6 muscle cell line originally developed by Yaffe (15) was used for these studies. Cells were grown in monolayer culture in 100-mm culture dishes (Lux) containing 10 ml of DMEM (Flow Laboratories, Rockville, Md.) supplemented with 10% fetal calf serum (Sterile Systems, Inc.) and gentamycin (50 μg/ml) in an atmosphere of 10% CO_2 and 90% air at 37°. Cells were routinely plated at 15,000–20,000 cells/cm^2, fed on day 2, and either subcultured on day 3 using 0.1% trypsin in phosphate-buffered saline, or harvested for preparation of membranes on day 5–6. The doubling time for preconfluent cells was 14 hr.

For experiments using intact cells in monolayer culture, cells were subcultured into 24-well, 16-mm cluster dishes (Costar) at a density of 20,000–25,000 cells/cm^2 and were used 3 days later. Cells between passages 58 and 87 were used for these studies, and no differences between cells from early or late passages were observed.

Validation of binding assays. In view of the number of unexpected properties of *beta*-adrenergic receptors observed, it was important to rule out artifacts that could have resulted from carrying out binding studies with intact cells in monolayer culture and to ensure that the interactions being measured occurred with *beta*-adrenergic receptors. An extensive series of experiments validating binding assays for all three tissue preparations included here has been performed (13, 14). In these experiments, effects of variations in cell number, protein concentration, and receptor concentration were investigated. Experiments to assess both equilibrium and kinetic properties of the receptor were carried out. Effects of varying the concentration of medium constituents were investigated, and the possibility that drug or radioligand was destroyed or taken up into cells during the assay was explored. The available evidence suggests that the assays used here are measuring the properties of *beta*-adrenergic receptors in both membrane preparations and intact cells (see refs. 13 and 14).

Binding assays for beta-adrenergic receptors on broken cells. Membranes from L6 cells were prepared by rinsing cells in monolayer culture three times with ice-cold 1 mM sodium/Hepes (pH 7.5), adding 10 ml of ice-cold 1 mM sodium/Hepes containing 2 mM EDTA to each 100-mm dish, and placing the dishes in an ice-water bath for 15 min to shock the cells osmotically. The cells were removed by scraping with a rubber policeman followed by homogenization with a Polytron, and the lysate was centrifuged at 20,000 × *g* for 10 min. The resulting pellets were resuspended by homogenizing with a Polytron in 1 mM sodium/Hepes containing 2 mM EDTA and centrifuged at 20,000 × *g* for 10 min. These pellets were resuspended by homogenization with a Polytron in 40 mM sodium/Hepes-buffered DMEM (usually containing 2% horse serum).

Hydroxybenzylpindolol was iodinated with carrier-free Na^{125}I (New England Nuclear Corporation). The [^{125}I]IHYP was purified by using descending paper chromatography to theoretical specific activity (2.2 Ci/μmole) as previously described (16).

The binding of [^{125}I]IHYP to membranes (5–10 μg of protein) prepared from L6 cells was carried out in a 37° water bath in disposable polypropylene tubes (Sarstedt). Equilibrium was reached by 40 min, and reactions were terminated after 50–70 min. The usual reaction contained 300 μM GTP, 75 μM phentolamine, 15 μg of bovine serum albumin, 2% horse serum, 40–65 pM [^{125}I]IHYP, and 40 mM sodium/Hepes (pH 7.5) in DMEM in a final incubation volume of 550 μl. The presence of medium, albumin, or serum in the binding reaction did not affect the results. Specific binding was defined as the amount of [^{125}I]IHYP bound in the absence of propranolol minus the amount bound in the presence of 0.3 μM (−)-propranolol. Filter blanks were usually less than 1% of the added radioligand. Specific binding routinely represented 90–95% of total binding.

Reactions were terminated by the addition of 10 ml of isotonic saline containing 10 mM Tris-HCl (pH 7.5; 20°), and samples were rapidly filtered through glass-fiber filters (no. 30, Schleicher and Schuell) on a Millipore filtration apparatus. The filters were dried under suction, and radioactivity was determined in a Beckman 4000 gamma counter.

Assays of beta-adrenergic receptors on intact cells in suspension. Attached cells (90,000–200,000/cm^2) were suspended after being washed

three times with 10 mM sodium/Hepes-isosaline (0.9% NaCl) at 37°. Cells were detached from plates following a 10-min incubation at 37° with 0.75 mM EDTA in 40 mM sodium/Hepes-isosaline, or a 3- to 5-min incubation at 37° with a solution of 0.025% trypsin in 40 mM sodium/Hepes-isosaline. DMEM containing 40 mM sodium/Hepes and 2% horse serum was then added, and the cells were suspended by gentle aspiration with a Pasteur pipette. Suspended cells were then used in binding assays. Incubation conditions were the same as those used to study receptors on membranes except for the addition of 2 mM $MgCl_2$ and 2 mM $CaCl_2$ following exposure of cells to EDTA. Residual trypsin was inactivated by the inclusion of 0.01% soybean trypsin inhibitor (Sigma Chemical Company). Equilibrium was reached by 45 min, and reactions were terminated after 50–70 min. Approximately 150,000–200,000 cells were used per assay, and specific binding represented about 80% of total binding.

Assays of beta-adrenergic receptors on intact cells in monolayer culture. Binding assays for intact cells in monolayer culture were performed in 24-well, 16-mm cluster dishes (Costar) as previously described (13). Each well contained approximately 250,000 cells, and variation in cell number between wells was routinely less than 5%. Prior to using cells for binding assays, the normal growth medium was removed from the wells and replaced with 500 μl of medium containing 40 mM sodium/Hepes (pH 7.5) and 2% horse serum. The cluster dishes were placed in an incubator at 37° for 5–6 hr before being used in binding assays. Cluster dishes were then placed in a 37° water bath, and binding reactions were initiated by adding [^{125}I]IHYP and various drugs in 10- or 20-μl aliquots. The reaction conditions were the same as those used to study receptors on membranes. Equilibrium was reached by 60–70 min, and reactions were terminated after 70–75 min. More than 95% cell viability (trypan blue exclusion and ability to grow after subculturing) was retained after exposure of cells to the reaction mixture at 37° for up to 15 hr. Assays were terminated by pouring off the reaction mixture and submerging the cluster dish in a beaker containing 10 mM Tris-isosaline (20°). The Tris-isosaline was poured off rapidly, and 0.8 ml of 10 mM sodium/Hepes-isosaline (20°) was added to each well. The cells in each well were then gently sonicated (less than 2 sec) with a Kontes cell disrupter. The contents of each well were quantitatively transferred to a Millipore filtration apparatus containing glass-fiber filters and washed with 20 ml of Tris-isosaline (pH 7.5, 20°). Specific binding routinely represented 70–80% of total binding.

Adenylate cyclase activity in membranes. Membrane preparations were prepared as described for binding assays except that only a single homogenization and centrifugation step was performed and the final resuspension was in 1 mM sodium/Hepes (pH 7.5). Adenylate cyclase activity was determined by measuring the amount of [^{32}P]cyclic AMP formed from α-^{32}P-labeled ATP according to modifications (17) of the method of Salomon *et al.* (18). The reaction mixture routinely contained 50 mM sodium/Hepes (pH 7.5), 0.5 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, 1.5 mM $MgCl_2$, 1 mM 1-methyl-3-isobutylxanthine, 0.25 mM ATP, 30 μM GTP, 20 mM creatine phosphate, creatine kinase (0.2 mg/ml), and [α-^{32}P]ATP (1–3 million cpm) prepared as described by Johnson and Walseth (19), and various drugs in a final incubation volume of 110 or 120 μl. Reactions were carried out at 37° for 8 min, and each assay contained about 30 μg of membrane protein. Under these conditions, the reaction was linear for 12–14 min. Recovery ranged between 50% and 70% for the entire procedure, and data have been corrected for recovery.

Cyclic AMP accumulation in intact cells. The accumulation of cyclic AMP was determined in intact cells under assay conditions identical with those used in studies of the binding of [^{125}I]IHYP except that in some cases phosphodiesterase activity was inhibited by preincubating cells with 1 mM 1-methyl-3-isobutylxanthine and 0.1 mM Ro 20-1724 for 45 min prior to initiation of the reaction. Values for K_{act} and K_i were determined following a 10-min incubation with drugs. The reaction was stopped by removing the medium from the cells and replacing it with 1 ml of 1 N HCl. The medium was placed in a boiling water bath for 5 min and used to determine extracellular levels of cyclic AMP. Aliquots of the acidic extracts containing 0.25–4 pmoles of intracellular cyclic AMP were evaporated to dryness using a Speed Vac concentrator (Savant). The residues were resuspended in 100 μl of Hepes-buffered medium and used to determine the intracellular content of cyclic AMP. Standards containing known amounts of cyclic AMP always accompanied unknown samples during preparative procedures.

Cyclic AMP concentrations in samples and standards were determined by the method of Brown *et al.* (20). Reactions were carried out in a final volume of 250 μl and contained 100 μl of unknown sample or cyclic AMP standard in boiled media, 8 mM sodium/Hepes (pH 7.4), 4.8 mM theophylline, 3.6 mM β-mercaptoethanol, [^{3}H]cyclic AMP (20,000–30,000 cpm, 36.4 Ci/mmole), and bovine adrenal binding protein (20). The reaction was initiated by adding the binding protein to tubes in an ice bath, and the reaction was allowed to proceed for 2–12 hr at 0°. Reactions were terminated by adding 100 μl of 100 mM Tris-HCl buffer (pH 7.4) containing 8 mM theophylline, 6 mM β-mercaptoethanol, charcoal (Norit SG "extra") (100 mg/ml), and bovine serum albumin (20 mg/ml). The samples were centrifuged at 1200 × *g* for 20 min, 175-μl aliquots of the supernatant were removed, and radioactivity was determined in a Triton X-100-based scintillation cocktail using a Searle Delta 300 scintillation counter.

Cyclic nucleotide phosphodiesterase activity. Cyclic nucleotide phosphodiesterase activity was determined by a modification of the procedure of Thompson and Appleman (21). Membranes prepared from L6 cells were obtained by rinsing 100-mm culture dishes twice with 40 mM sodium/Hepes-buffered medium (pH 7.5) at room temperature, followed by addition of 10 ml of 5 mM sodium/Hepes containing 0.1% Triton X-100. Cells were removed from the plates with a rubber policeman and homogenized with a Brinkmann Polytron (setting 5 for 10 sec). Homogenates were then diluted 20-fold with 40 mM sodium/Hepes-buffered medium, and 80 μl were used in each assay. The final incubation volume was 100 μl and contained, in addition to medium constituents, 2–3 μg of protein, 40 mM sodium/Hepes (pH 7.5), 5 mM $MgCl_2$, 70–80 pmoles of cold cyclic AMP, and 100,000–200,000 cpm of [^{3}H]cyclic AMP. The reaction was initiated by the addition of tissue and placing the tubes in a 30° water bath for 10 min. This step of the assay allowed endogenous tissue phosphodiesterases to convert [^{3}H]cyclic AMP to [^{3}H]5′-AMP. The reaction was terminated by placing the tubes in a boiling water bath for 2.5 min. The tubes were allowed to cool to 20°, followed by addition of 0.05 mg of snake venom (*Crotalus atrox*; Sigma Chemical Company) in 100 μl of water. The tubes were incubated at 30° for an additional 10 min, during which time 5′-AMP was converted to adenosine. The reaction was stopped by adding 500 μl of a 1:4 slurry of Bio-Rad anion exchange resin, AG1-X2, 200–400 mesh, Cl^- form in water. The tubes were agitated with a Vortex mixer and the resin was allowed to settle for 10 min, after which samples were centrifuged at 1000 × *g* for 5 min. The anion exchange resin bound all charged nucleotides, whereas [^{3}H]adenosine remained in the supernatant. A 250-μl aliquot of the supernatant was counted in a Triton X-100-based scintillation cocktail.

Protein determination. Protein was determined by the method of Bradford (22), using bovine serum albumin as a standard.

Data analysis. All analyses were performed using a WANG 2200-T computer system. Nonweighted linear regression analysis was performed for Scatchard and Hill plots. The K_D values for inhibition of [^{125}I]IHYP binding and K_i values for inhibition of isoproterenol-stimulated cyclic AMP accumulation by various drugs were calculated using the following equation (23): $K_D = IC_{50}/(1 + S/K_m)$, where IC_{50} is the concentration of drug which inhibited 50% of the specific [^{125}I]IHYP binding or isoproterenol-stimulated cyclic AMP accumulation, S is the concentration of [^{125}I]IHYP (binding) or isoproterenol (adenylate cyclase), and K_m is the K_D value for [^{125}I]IHYP determined by Scatchard analysis (24) or the K_{act} value for stimulation of cyclic AMP accumulation by isoproterenol. It has been shown empirically that, for *beta*-adrenergic receptors, the apparent K_D value of a drug determined by inhibition of binding of [^{125}I]IHYP as calculated by the method of Cheng and Prusoff (23) is independent of the Hill coefficient (6). Therefore, this equation can be applied even when the Hill coefficient for inhibition of binding of [^{125}I]IHYP is significantly less than 1.0. In

TABLE 1

Activation and inhibition of adenylate cyclase activity in intact cells and membranes

Duplicate determinations were performed using 10 (intact cells) or 13 (membranes) concentrations of each drug. Determinations of basal activity and maximal stimulation (50 μM (−)-isoproterenol) were included with each dose-response curve. The intrinsic activity for agonists is given relative to that of isoproterenol. The average stimulation by isoproterenol was 221 ± 14 pmoles of cyclic AMP formed per minute per milligram of protein and 132 ± 27 pmoles of intracellular cyclic AMP per 10^6 cells for membranes and intact cells, respectively. Typically, isoproterenol produced a 5- to 10-fold stimulation of adenylate cyclase activity in membranes and a 300- to 500-fold stimulation of cyclic AMP accumulation in studies with intact cells. K_i values for antagonists were determined from studies of the inhibition of isoproterenol-stimulated (0.025 μM) cyclic AMP accumulation. Values represent means ± standard error of the mean. The number of experiments is given in parentheses after each drug for intact cells and membranes, respectively. When only one number is given, the same number of experiments was performed for intact cells and for membranes.

	K_{act} or K_i		Intrinsic activity	
	Cells	Membranes	Cells	Membranes
	nM			
Full agonists				
Isoproterenol (n = 6, 10)	25 ± 9	82 ± 17	1.0	1.0
Cc-25 (n = 3)	2.4 ± 1.0	23 ± 13	0.98 ± 0.02	0.96 ± 0.06
Fenoterol (n = 3)	31 ± 8	140 ± 60	1.04 ± 0.05	1.03 ± 0.02
Epinephrine (n = 3)	38 ± 6	160 ± 70	0.95 ± 0.05	1.02 ± 0.03
Norepinephrine (n = 3)	2,540 ± 960	3,210 ± 840	1.02 ± 0.01	1.00 ± 0.02
Metaproterenol (n = 3)	1,360 ± 430	2,790 ± 230	1.03 ± 0.01	0.95 ± 0.02
Terbutaline (n = 3, 5)	720 ± 280	1,810 ± 140	1.01 ± 0.05	0.89 ± 0.04
OPC 2009 (n = 3)	5.7 ± 0.7	25 ± 13	1.17 ± 0.09	1.08 ± 0.05
Zinterol (n = 3, 5)	1.2 ± 0.5	8.4 ± 2.0	1.00 ± 0.00	0.91 ± 0.02
Salmefamol (n = 3, 4)	13 ± 4	47 ± 4	0.97 ± 0.03	0.97 ± 0.01
Partial agonists				
Salbutamol (n = 3)	190 ± 80	340 ± 110	0.89 ± 0.02	0.84 ± 0.03
Soterenol (n = 3, 4)	80 ± 55	140 ± 30	0.84 ± 0.03	0.76 ± 0.05
Carbuterol (n = 3)	480 ± 80	550 ± 60	1.00 ± 0.03	0.65 ± 0.04
Dobutamine (n = 3)	4,610 ± 370	3,030 ± 1,480	0.57 ± 0.06	0.63 ± 0.00
Sulfonterol (n = 3)	82 ± 12	98 ± 20	0.29 ± 0.06	0.26 ± 0.03
Ephedrine (n = 3)	20,800 ± 4,000	5,830 ± 1,260	0.23 ± 0.01	0.25 ± 0.06
Antagonists				
Pindolol (n = 3)	1.9 ± 0.8	2.1 ± 0.3		
Butoxamine (n = 3)	3,250 ± 830	1,320 ± 610		
Practolol (n = 2)	38,100 ± 5,400	48,300 ± 5,300		
Atenolol (n = 2)	15,700 ± 3,300	12,050 ± 1,040		
Sotalol (n = 2)	1,380 ± 280	560 ± 110		
Timolol (n = 2)	0.4 ± 0.1	0.3 ± 0.1		
Propranolol (n = 3)	2.3 ± 0.8	1.3 ± 0.3		
Metoprolol (n = 3)	2,110 ± 1,350	1,930 ± 390		

this case the Cheng and Prusoff equation yields a $K_{0.5}$ value that reflects the concentration of ligand which saturates one-half of the total number of binding sites. In the limiting case with a Hill coefficient of 1, the $K_{0.5}$ becomes identical with the K_D.

RESULTS

Determination of intrinsic activities. Sixteen agonists at *beta*-adrenergic receptors were studied to determine their intrinsic activities relative to that of isoproterenol in membrane preparations and intact cells. Intrinsic activities ranged from 0.23 to 1.17 (Table 1). An arbitrary criterion was established that a full agonist was a drug with an average intrinsic activity (intact cells and membranes) greater than 0.90. According to this criterion, 10 of the 16 drugs were classified as full agonists (Table 1). With the exception of carbuterol, all agonists showed similar intrinsic activities in intact cells and in membranes. Carbuterol was as efficacious as isoproterenol in intact cells, but only 65% as efficacious in membranes. Both full and partial agonists appeared to stimulate adenylate cyclase only through interactions with *beta*-adrenergic receptors. The antagonist propranolol (1 μM) quantitatively inhibited adenylate cyclase stimulation by these agonists at their EC_{50} concentrations (data not shown). The K_i values of eight antagonists were also determined (Table 1). None of these compounds stimulated adenylate cyclase activity in membranes or cyclic AMP accumulation in intact cells.

Inhibition of the binding of [^{125}I]IHYP by antagonists. The ability of eight antagonists to inhibit the binding of [^{125}I]IHYP in broken and intact cells was determined (Table 2). Inhibition of the binding of [^{125}I]IHYP appeared to follow simple competitive kinetics with pseudo-Hill coefficients (n_H) of approximately 1. The potencies of antagonists were the same in intact cells and in membranes, as indicated by potency ratios (K_D cell/K_D membrane) of approximately 1 (range 0.5–1.2; Table 2).

Inhibition of the binding of [^{125}I]IHYP by agonists. A large range of $K_{0.5}/K_{act}$ ratios was observed in studies of the interactions of agonists with receptors on intact cells (Table 2). Weak partial agonists (ephedrine, sulfonterol, and dobutamine) were characterized by $K_{0.5}/K_{act}$ ratios of about 1 (Table 2), whereas more efficacious partial

TABLE 2

Inhibition of [^{125}I]IHYP binding in intact cells in monolayer culture and membrane preparations by agonists and antagonists

Numbers in parentheses following drugs indicate the number of experiments performed in intact cells in monolayer culture and membranes, respectively. Duplicate asssays were performed in each experiment, and 10 (intact cells) and 16 (membranes) concentrations of drugs were used. Pseudo-Hill coefficients (n_H) were determined from Hill plots of the data. Values represent means ± standard error of the mean. GTP (300 μM) was included in assays using membranes. Values for K_{act} and K_i (last column) were taken from Table 1. Values for $K_{0.5}$ represent the concentrations of drugs which saturate one-half of the total number of binding sites.

	Cells		Membranes		$K_{0.5}$ cell/K_D membrane	$K_{0.5}$ cell/K_{act} or K_i
	$K_{0.5}$	n_H	K_D	n_H		
	μM		μM			
Agonists						
Group I						
Isoproterenol (10, 16)	43.6 ± 2.35	1.04 ± 0.08	0.753 ± 0.046	0.91 ± 0.03	57.9	1744
Cc-25 (10, 5)	6.41 ± 0.76	1.01 ± 0.09	0.093 ± 0.005	0.89 ± 0.05	68.9	2671
Fenoterol (8, 5)	25.0 ± 1.65	0.98 ± 0.05	1.09 ± 0.043	0.94 ± 0.01	22.9	806
Epinephrine (5, 5)	136 ± 34	0.97 ± 0.03	3.47 ± 0.13	0.87 ± 0.02	39.2	3579
Norepinephrine (4, 6)	627 ± 166	1.03 ± 0.06	37.76 ± 2.85	0.90 ± 0.04	16.6	247
Group II						
Metaproterenol (8, 5)	160 ± 31.9	0.52 ± 0.05	24.47 ± 2.60	1.01 ± 0.04	6.5	118
Terbutaline (7, 7)	55.8 ± 6.0	0.67 ± 0.09	11.86 ± 1.44	0.93 ± 0.02	4.7	78
OPC 2009 (8, 7)	0.34 ± 0.05	0.84 ± 0.03	0.111 ± 0.004	1.01 ± 0.02	3.1	60
Salbutamol (10, 6)	3.56 ± 0.68	0.74 ± 0.08	1.17 ± 0.26	0.94 ± 0.03	3.0	19
Soterenol (7, 6)	1.81 ± 0.49	0.60 ± 0.06	0.596 ± 0.058	1.02 ± 0.04	3.0	23
Carbuterol (10, 5)	5.57 ± 0.76	0.49 ± 0.01	1.62 ± 0.11	0.94 ± 0.03	3.4	12
Group III						
Zinterol (8, 7)	0.031 ± 0.006	0.92 ± 0.05	0.025 ± 0.0022	0.97 ± 0.07	1.2	26
Salmefamol (6, 6)	0.40 ± 0.05	0.89 ± 0.06	0.457 ± 0.037	0.99 ± 0.06	0.9	31
Dobutamine (6, 5)	5.12 ± 0.40	1.02 ± 0.06	4.57 ± 0.05	1.00 ± 0.02	1.1	1
Sulfonterol (6, 5)	0.099 ± 0.008	0.75 ± 0.06	0.132 ± 0.037	0.97 ± 0.02	0.8	1
Ephedrine (8, 6)	10.40 ± 0.42	1.00 ± 0.05	12.17 ± 0.75	0.96 ± 0.02	0.9	0.5
Antagonists						
Pindolol (6, 4)	0.0008 ± 0.0001	1.02 ± 0.10	0.00083 ± 0.00008	1.01 ± 0.03	1.0	0.4
Butoxamine (4, 4)	0.98 ± 0.25	0.96 ± 0.03	0.96 ± 0.20	0.97 ± 0.02	1.0	0.3
Practolol (4, 4)	33.66 ± 9.3	0.98 ± 0.03	34.39 ± 4.37	0.97 ± 0.01	1.0	0.9
Atenolol (6, 4)	10.98 ± 0.68	1.01 ± 0.01	9.97 ± 1.34	1.00 ± 0.04	1.1	0.7
Sotalol (4, 4)	0.62 ± 0.09	0.93 ± 0.06	0.52 ± 0.08	1.01 ± 0.03	1.2	0.4
Timolol (4, 4)	0.00016 ± 0.0001	0.94 ± 0.04	0.0003 ± 0.00005	0.98 ± 0.03	0.5	0.4
Propranolol (6, 5)	0.0011 ± 0.0003	0.98 ± 0.03	0.0010 ± 0.0003	1.00 ± 0.01	1.1	0.5
Metoprolol (5, 4)	1.14 ± 0.28	0.99 ± 0.05	1.03 ± 0.13	0.95 ± 0.04	1.1	0.5

agonists (salbutamol, soterenol, and carbuterol) were characterized by ratios of 10–20. The $K_{0.5}/K_{act}$ ratios for full agonists ranged between 26 for zinterol and 3600 for epinephrine. The observation that some full agonists were characterized by low $K_{0.5}/K_{act}$ ratios was unexpected in view of the large ratios observed in studies of the interactions of a limited number of full agonists with *beta*-adrenergic receptors on intact S49 (11) and C6-2B (10) cells.

Unlike the data obtained with antagonists, a considerable range of potency ratios ($K_{0.5}$ cell/K_D membrane) was observed for the 16 agonists tested (Table 2). Five drugs (Group I), all of which were full agonists, had potency ratios of 17–69. Six agonists (Group II) had ratios between 3 and 7. This group included three partial agonists (salbutamol, soterenol, and carbuterol) and three full agonists (metaproterenol, terbutaline, and OPC 2009). The other five drugs (Group III), including three weak partial agonists (dobutamine, sulfonterol, and ephedrine) and two full agonists (zinterol and salmefamol), had potency ratios of about 1. Thus, agonists could be subdivided into three groups according to the magnitude of the ratio of the $K_{0.5}$ values determined in intact cells in monolayer culture and the K_D values determined in membranes. Inhibition curves of the binding of [^{125}I]IHYP to intact cells and membranes in the presence of GTP by one drug from each group are shown in Fig. 1. The inhibition curves for zinterol were almost superimposable (Fig. 1), suggesting that zinterol was binding to the same form of the *beta*-adrenergic receptor in intact cells and in membranes in the presence of GTP. Conversely, receptors on intact cells had a much lower affinity for isoproterenol than was observed in studies with membranes. Data from a previous study (13) suggested that this resulted from the conversion of receptors on intact cells to a form with a low affinity for agonists. The curves describing inhibition of the binding of [^{125}I]IHYP by metaproterenol were unusual in that the inhibition curves obtained using intact cells were shallow. An examination of pseudo-Hill coefficients of those drugs whose interactions were characterized by intermediate potency ratios (3–7) indicated that all six of these Group II drugs had low pseudo-Hill coefficients in intact cells (0.49–0.84; Table 2), whereas in membranes in the presence of GTP all of these agonists had pseudo-Hill coefficients close to 1 (range 0.93–1.02; Table 2). The relationship between the pseudo-Hill coefficients in intact cells and the potency ratios is shown in Fig. 2. Those

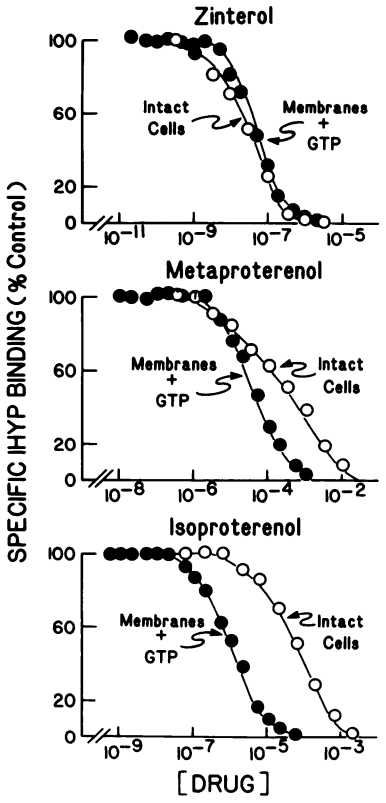

FIG. 1. *Inhibition of the binding of [^{125}I]IHYP to receptors on intact cells in monolayer culture and on membranes*

Assays were performed in duplicate using either 10 (intact cells, ○) or 16 (membranes, ●) concentrations of each drug as described under Materials and Methods. Assays with membranes were carried out in the presence of 300 μM GTP.

drugs having potency ratios of 1 had pseudo-Hill coefficients of approximately 1 and those drugs with large potency ratios also had pseudo-Hill coefficients of 1 (Table 2; Fig. 2). Low pseudo-Hill coefficients associated with drugs with intermediate ratios (Group II) could be interpreted as resulting from interactions of agonists with two forms of the receptor with different affinities for the agonist. Thus, exposure of receptors on intact cells to these drugs appears to result in the conversion of some but not all of the receptors from a form with a high affinity for agonists to a form with a low affinity.

Initial interactions of isoproterenol and zinterol with beta-adrenergic receptors on intact cells. In agreement with previous results (13), it was observed that 5 μM isoproterenol inhibited the specific binding of [^{125}I]IHYP to *beta*-adrenergic receptors on intact cells for 2 min (Fig. 3) but had no effect on the binding of [^{125}I]IHYP at times longer than 10 min. This concentration of isoproterenol is sufficient to inhibit approximately 90% of the specific binding of [^{125}I]IHYP in membrane preparations throughout the time course of binding. In contrast, zinterol (Fig. 3) as well as salmefamol (data not shown) inhibited binding of [^{125}I]IHYP in an apparently competitive manner from 1–70 min (Fig. 3). Thus, the affinity of receptors on intact cells for isoproterenol appeared to decrease during the assay, whereas the affinity of the receptor for zinterol did not change during the reaction.

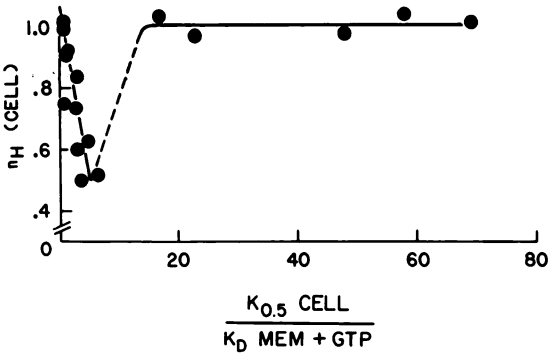

FIG. 2. *Relationship between pseudo-Hill coefficients for agonists in intact cells in monolayer culture and the ratio of $K_{0.5}$ values in cells to K_D in membranes*

Data were taken from Table 2. Results obtained in studies of 10 full agonists and 6 partial agonists are shown. Assays of receptors on membranes were carried out in the presence of 300 μM GTP.

Isoproterenol-stimulated rates of cyclic AMP accumulation and extrusion in intact cells. Upon exposure of cells to 10 μM (−)-isoproterenol, a rapid initial rate of accumulation of cyclic AMP occurred, followed by a subsequent decline in the rate after 1–2 min (Fig. 4). The decreased rate of accumulation was observed in the absence (Fig. 4, *top*) and presence (Fig. 4, *bottom*) of phosphodiesterase inhibitors and was, therefore, not the result of activation of phosphodiesterases. Direct measurement of phosphodiesterase activity indicated that it was the same in control cells as in cells exposed to isoproterenol for 5 min (52.9 ± 3.1 and 50.8 ± 1.1 pmoles of cyclic AMP converted per minute per milligram of protein for control cells and cells exposed to isoproterenol, respectively; n = 3 plates for each determination). The biphasic rates of intracellular cyclic AMP accumulation were not the result of a change in the rate of extrusion of cyclic AMP into the extracellular medium (Fig. 4).

Intracellular cyclic AMP accumulation following exposure of cells to isoproterenol, zinterol, and salmefamol. The rates of cyclic AMP accumulation following exposure to maximally effective concentrations of isopro-

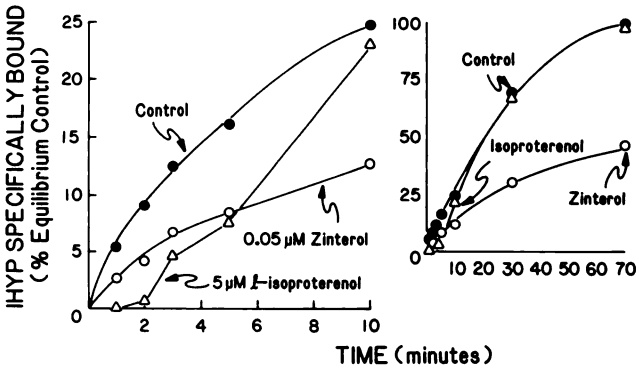

FIG. 3. *Time course of specific [^{125}I]IHYP binding in control cells and in the presence of isoproterenol or zinterol*

Reactions performed in triplicate as described under Materials and Methods were initiated by adding [^{125}I]IHYP (50–60 pM) and media (●), isoproterenol (5 μM, △), or zinterol (0.05 μM, ○) in 20-μl aliquots to cells in monolayer culture. The data shown were combined from two similar experiments.

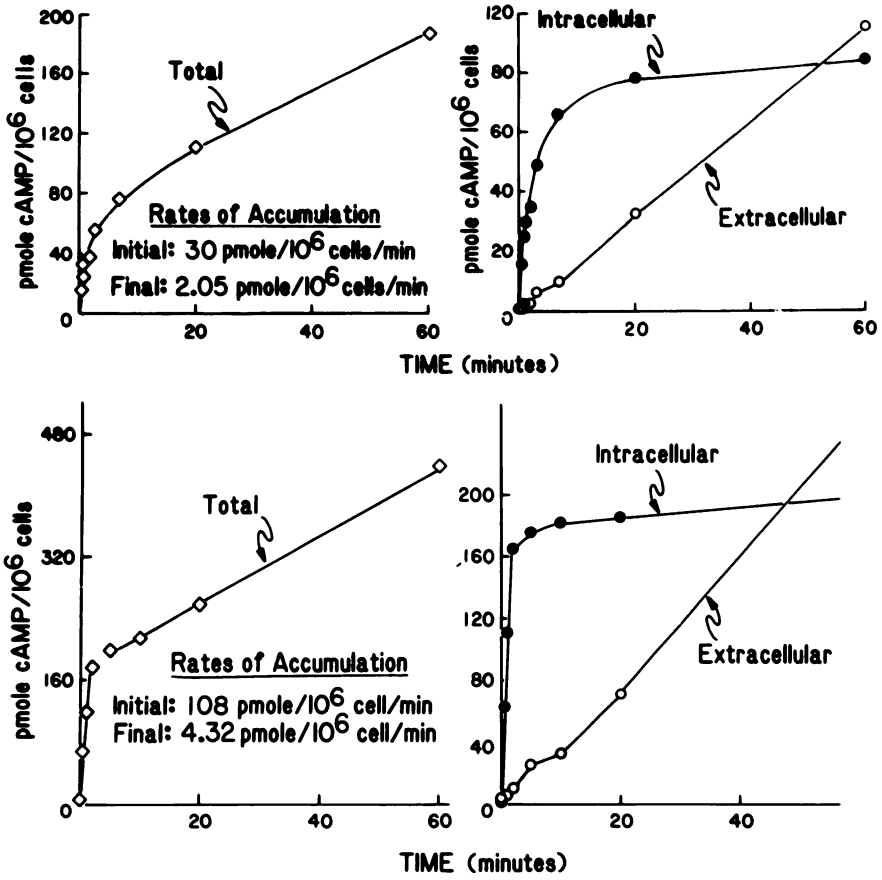

FIG. 4. *Isoproterenol-stimulated cyclic AMP accumulation in intact cells in the absence and presence of phosphodiesterase inhibitors*

Assays were performed in duplicate or triplicate in 24-well cluster dishes. Reaction conditions and methods for determination of intracellular (●) and extracellular (○) levels of cyclic AMP are described under Materials and Methods. Estimates of the initial rate (0–1 min) and final rate (10–60 min) of total cyclic AMP accumulation are shown. Reactions were initiated by addition of 10 μM isoproterenol. *Top.* Cyclic AMP accumulation in the absence of a phosphodiesterase inhibitor. The data are representative of four similar experiments. *Bottom.* Cells were preincubated for 45 min with 1 mM 1-methyl-3-isobutylxanthine and 0.1 mM Ro 20-1724 to inhibit phosphodiesterase activity. The data are representative of three similar experiments.

terenol (10 μM), zinterol (1 μM), and salmefamol (3 μM) were measured. The rates of cyclic AMP accumulation observed were approximately the same in the presence of all three drugs (Fig. 5). Thus, the kinetics of cyclic AMP accumulation were the same for a drug that converted receptors on intact cells to a low-affinity form (isoproterenol) as for two drugs that did not convert receptors to a low-affinity form (zinterol and salmefamol). The rate of extrusion of cyclic AMP was also the same following exposure of cells to the three drugs (data not shown).

Properties of beta-adrenergic receptors on suspended intact L6 cells. The pharmacological properties of *beta*-adrenergic receptors on intact L6 cells in suspension were the same as those for receptors on membranes assayed in the presence of GTP (Table 3). The properties did not resemble those observed for receptors on intact cells in monolayer culture (Table 3). This was an unexpected finding, since cells in suspension were still viable following a 70-min binding assay as evidenced by the ability of more than 90% of the cells to exclude trypan blue and to attach and begin dividing following transfer to a culture dish. Similar pharmacological properties of *beta*-adrenergic receptors were observed following detachment of cells with either EDTA or trypsin; therefore, the data for the two groups have been combined in Table 3.

To study the affinity of *beta*-adrenergic receptors on

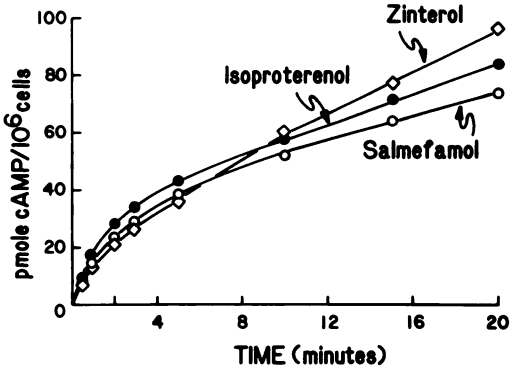

FIG. 5. *Time course of isoproterenol-, zinterol-, and salmefamol-stimulated cyclic AMP accumulation*

Assays of intracellular cyclic AMP accumulation were performed in duplicate in 24-well cluster dishes as described under Materials and Methods. Reactions were initiated by the addition of zinterol (1 μM, ◇), salmefamol (3 μM, ○), or isoproterenol (10 μM, ●). The experiment was performed three times, and the data presented are from a representative experiment.

TABLE 3

Comparison of the properties of beta-adrenergic receptors on membrane preparations, intact L6 cells in suspension, and intact cells in monolayer culture

The results shown are K_D or $K_{0.5}$ values determined either by Scatchard analysis of the binding of [^{125}I]IHYP or by inhibition of the binding of [^{125}I]IHYP by 8–16 concentrations of each competing ligand. The concentration of GTP in binding assays with membranes was 300 μM. Values shown are means ± standard error of the mean for 3–12 independent determinations except for intact cell values for (+)-isoproterenol, which are the averages of two inhibition curves from a single experiment. Some of the data for membranes and intact cells in monolayer culture were taken from Table 1. Most of the data for intact cells in suspension were obtained from cells detached with EDTA; however, the data obtained from cells detached with trypsin were the same, so the data have been combined.

	Membranes	Intact cells	
		Suspension	Monolayer
[^{125}I]IHYP (pM)	46 ± 4	55 ± 6	43 ± 4
(−)-propranolol (nM)	1.0 ± 0.3	1.1 ± 0.1	1.1 ± 0.3
(+)-propranolol (nM)	145 ± 19	94 ± 8	101 ± 14
Practolol (μM)	34 ± 4	61 ± 14	34 ± 9
(−)-isoproterenol (μM)			
+ GTP	0.7 ± 0.12	0.88 ± 0.09	47 ± 4
− GTP	0.029 ± 0.004	1.0 ± 0.21	47 ± 2
(+)-isoproterenol (+ GTP; μM)	57 ± 8	81	>500
(−)-epinephrine (+ GTP; μM)	3.47 ± 0.13	2.66 ± 0.27	136 ± 34
(−)-norepinephrine (+GTP; μM)	38 ± 3	23 ± 5	627 ± 166
Zinterol (+ GTP; nM)	25 ± 2	22 ± 7	31 ± 6
Salmefamol (+ GTP; nM)	457 ± 37	496 ± 48	400 ± 50
Soterenol (+ GTP; μM)	0.6 ± 0.06	0.51 ± 0.08	1.8 ± 0.5

intact cells in suspension and monolayer culture under exactly the same conditions and in the same reaction mixture, advantage was taken of the fact that the initial attachment of the cells to the dish is via adsorption (25). Pretreatment of dishes with bovine serum albumin (100 μg/well) reduced the rate of cell attachment. Cells were added to cluster dishes treated with albumin and preincubated for 15 min at 37°, after which [^{125}I]IHYP and varying amounts of isoproterenol were added and the incubation was continued for an additional 60 min. During this 75-min period, about 20% of the cells attached and began flattening out while the remainder stayed in suspension. The reaction mixture was removed, and the amount of [^{125}I]IHYP bound to cells in suspension was determined. Attached cells and [^{125}I]IHYP bound to them were then removed by brief sonication. In this way, the properties of cells in suspension and newly attached cells were determined simultaneously in the same wells. The potency of isoproterenol for inhibition of [^{125}I]IHYP binding was much lower for cells that had attached than for those in suspension (K_D = 0.89 ± 0.26 μM and 81 ± 30 μM for suspended and attached cells, respectively; n = 6).

The concentrations of trypsin and EDTA used to suspend cells did not appear to affect *beta*-adrenergic receptors directly. The K_D values for isoproterenol were the same in control membrane preparations and membrane preparations assayed in the presence of 0.025% trypsin or 1 mM EDTA (K_D = 0.21 ± 0.03, 0.19 ± 0.01, and 0.21 ± 0.01 μM for control, trypsin-containing, and EDTA-containing assays; n = 3 for each condition).

Exposure of cells in monolayer culture to EDTA or trypsin did not appear to alter the affinity of the receptors for agonists. When cells in monolayer culture were exposed for short periods of time to EDTA or trypsin, the cells were morphologically altered (the cells became rounded in shape and separated from one another) but did not lift off the dishes. The potency of isoproterenol for inhibition of [^{125}I]IHYP binding in these cells was the same as in control cells (Table 4). The number of receptors per cell was not altered following suspension of the cells (20,167 ± 1249 compared with 19,728 ± 1192 receptors/cell for monolayer and suspended cells, respectively; n = 3). Therefore, treatment with EDTA or trypsin did not selectively destroy a population of receptors present on cells in monolayer culture. Cells detached using either EDTA or trypsin had similar pharmacological properties; thus the properties of suspended cells did not appear to result from the method used to detach cells.

Cells in suspension and cells in monolayer culture have very different morphologies. Cells attached to a substratum have a highly structured cytoskeleton. Nearly all of this organization is lost when cells detach. Moreover, it has been shown that agents that disrupt cytoskeletal elements affect the mobility (26) and function (27, 28) of *beta*-adrenergic receptors. For these reasons, it was of interest to determine whether cytoskeletal disrupting agents would affect the ability of isoproterenol to convert *beta*-adrenergic receptors to a form with a low affinity for agonists. Receptors on cells treated with the microtubule-disrupting agent colchicine or the microfilament-disrupting agent cytochalasin B had the same affinity for isoproterenol as receptors on untreated cells (Table 4). Therefore, microtubules and microfilaments did not appear to be necessary for the production or maintenance of the low-affinity form of *beta*-adrenergic receptors on intact cells.

The possibility that physical contact between the cell and the substratum affected the properties of the membrane so as to facilitate the appearance of a form of the receptor with a low affinity for agonists was investigated.

TABLE 4

Potency of isoproterenol for inhibition of [^{125}I]IHYP binding in intact cells in monolayer culture following various treatments

Numbers in parentheses following treatments indicate the number of inhibition curves used in determining $K_{0.5}$ values for isoproterenol. Cells in cluster dishes were treated for 2 min with 0.5 mM EDTA or for 1 min with 0.05% trypsin, after which these solutions were removed and the reaction mixture added to the wells. Cells were "fixed" in cluster dishes by freezing cells in 40 mM sodium/Hepes medium (pH 7.5) at −70° for 3 hr followed by 1 hr at 4° and 1 hr at 21°. The cells were killed but remained attached to the dishes and maintained an apparently normal morphological shape. Cells in cluster dishes were treated with colchicine (100 μM final concentration) for 45 min or with cytochalasin B (10 μg/ml final concentration) for 20 or 180 min prior to initiating reactions with [^{125}I]IHYP and isoproterenol. Data for the two cytochalasin B groups were not different and have been combined. Assays were carried out as described under Materials and Methods.

Treatment	$K_{0.5}$ isoproterenol
	μM
Control (n = 13)	41 ± 3
EDTA (n = 3)	38 ± 4
Trypsin (n = 3)	40 ± 3
"Fixed" cells (n = 3)	0.19 ± 0.03
Colchicine (n = 9)	46 ± 4
Cytochalasin B (n = 12)	59 ± 11

Cells in monolayer culture that had been killed by freezing had *beta*-adrenergic receptors with a high affinity for isoproterenol (Table 4) even though the cells were attached to the dishes and had a normal morphological appearance. Therefore, physical contact with the dish was not in itself sufficient to allow formation of the low-affinity state of the receptor in the presence of isoproterenol.

DISCUSSION

A number of unexpected observations with regard to the interactions of agonists with *beta*-adrenergic receptors on intact cells have been described. Certain agonists have the ability to induce a form of the receptor on intact cells which has a low affinity for agonists. This effect was seen in studies of cells attached to the substratum but not in cells that had been suspended. A series of experiments has been performed to ensure that *beta*-adrenergic receptors are being measured, and the data are not a result of artifacts associated with performing binding studies with intact cells (see refs. 13 and 14). In addition, data obtained with eight antagonists were compared. Excellent correlations (r = 0.99 for each comparison) were obtained for K_D values in attached cells and K_D values in membranes, K_i values in attached cells and K_i values in membranes, or K_D values and K_i values for attached cells (data from Tables 1 and 2). Therefore, for antagonists, the binding assays that have been used are measuring the same site in membrane preparations and in intact attached cells, and this binding site is also the site through which adenylate cyclase is activated.

Previous studies of the properties of *beta*-adrenergic receptors on intact cells revealed unexpectedly low affinities for isoproterenol (10, 11, 13) as well as for epinephrine and norepinephrine (13). In the current experiments, the effects of a large number of agonists ranging from weak partial agonists to full agonists were investigated. The ratios of the $K_{0.5}$ values obtained in studies with intact cells to K_D values obtained in studies with membranes in the presence of GTP (potency ratios) were determined. Full agonists such as zinterol and salmefamol had potency ratios of 1, whereas other full agonists, such as isoproterenol and Cc-25, had potency ratios greater than 50. Partial agonists had potency ratios ranging from 1 to 3. Therefore, there was no simple correlation between the efficacy of an agonist and its potency ratio. Similarly, there was no correlation between the potency ratios and the K_{act} values for stimulation of adenylate cyclase activity or the accumulation of cyclic AMP.

Pseudo-Hill coefficients were determined from analysis of competition curves of agonist inhibition of the binding of [^{125}I]IHYP in intact cells. Agonists having low potency ratios as well as agonists having very high potency ratios were characterized by pseudo-Hill coefficients of 1. This indicated that only one form of the receptor existed in the presence of these drugs. Drugs such as zinterol, salmefamol, dobutamine, and ephedrine interacted with receptors on attached and disrupted cells in the same manner as evidenced by superimposable inhibition curves. Agonists such as isoproterenol, Cc-25, fenoterol, epinephrine, and norepinephrine appeared to bind to receptors on attached cells and to convert all of the receptors to a form having a low affinity for agonists. Therefore, inhibition curves by agonists in both groups were characterized by pseudo-Hill coefficients of 1.

Several lines of evidence support the theory that the properties of receptors on intact cells attached to the substriatum are similar to, if not identical with, the properties of receptors on membranes when assayed in the presence of GTP, and that some but not all agonists convert *beta*-adrenergic receptors on attached cells to a form having a low affinity for agonists. Previous work (13) had shown that *beta*-adrenergic receptors on intact cells in monolayer culture initially had approximately the same affinity for isoproterenol, epinephrine, and norepinephrine as did receptors on membranes when studied in the presence of GTP. After 1–2 min of exposure to these agonists, the receptors on intact cells appeared to be converted to a form which had the same affinity for antagonists but a very low affinity for agonists. In the present study, 5 μM (−)-isoproterenol was shown to inhibit the binding of [^{125}I]IHYP to intact cells in monolayer culture almost completely for about 2 min, after which it did not appear to inhibit binding, and at equilibrium no effect was observed on the amount of [^{125}I]IHYP bound. The same concentration of isoproterenol would have inhibited the binding of [^{125}I]IHYP to *beta*-adrenergic receptors on membranes by approximately 90% if assays were carried out in the presence of GTP. In the same experiment, the full agonists salmefamol and zinterol did not inhibit the binding of [^{125}I]IHYP in attached cells in a manner similar to that observed for isoproterenol, but rather inhibited the binding of the radioligand in a simple competitive manner throughout the time course of the reaction (Fig. 3). This suggested that zinterol and salmefamol did not convert receptors on intact cells to a new form with a reduced affinity for agonists.

Additional lines of evidence suggest that receptors on intact cells attached to the substratum were being converted from a form with a high affinity for agonists to a form with a relatively low affinity. In equilibrium binding experiments, agonists such as zinterol and salmefamol apparently interacted with the same form of the receptor on intact cells and on membranes, whereas agonists such as isoproterenol and epinephrine converted this form of the receptor on intact cells to one which had a low affinity for agonists. Also, the pseudo-Hill coefficients of inhibition curves in attached cells for agonists with intermediate potency ratios ranged from 0.49 to 0.84. A likely interpretation of these shallow inhibition curves is that two forms of the receptor were present with different affinities for the agonist. This would be expected if some but not all of the receptors had been converted to a form having a low affinity for agonists. Therefore, interactions of these drugs would represent the transition situation in receptor states between that observed in studies with agonists such as zinterol and that produced by agonists such as isoproterenol.

Studies of the time course of isoproterenol-stimulated cyclic AMP accumulation revealed marked decreases in the rate of cyclic AMP formation that occurred after only 2 min of exposure to an agonist. The decreased rate did not appear to be the result of activation of phosphodiesterases or extrusion of cyclic AMP into the medium. The

decrease in the rate of accumulation occurred at about the same time as the agonist-induced conversion of the receptors to a form having a low affinity for agonists. The possibility that the decrease in the affinity of receptors for isoproterenol was responsible for the decrease in the rate of cyclic AMP formation was investigated. Cells were exposed to zinterol or salmefamol, drugs that did not convert receptors to a low-affinity form. However, a marked reduction in the rate of cyclic AMP formation was observed. The reduction in rate occurred at the same time as the reduction observed when assays were carried out in the presence of isoproterenol. Furthermore, the abrupt decrease in the rate of cyclic AMP formation occurred even when assays were carried out in the presence of concentrations of isoproterenol (300 μM) capable of binding to most of the receptors even though they had a low affinity for isoproterenol. Therefore, conversion of receptors to a form with a low affinity for agonists did not appear to be responsible for the decrease in the rate of cyclic AMP production. It is possible that the molecular events leading to or associated with a decrease in the rate of cyclic AMP formation might also allow drugs such as isoproterenol to convert the receptors to a form having a low affinity for agonists.

At present, one can only speculate concerning the molecular basis of the low-affinity form of the receptor observed in intact cells in monolayer culture. Likely possibilities include specific phosphorylations or methylations, which occur in the presence of agonists such as isoproterenol but not in the presence of agonists like zinterol. Specific changes in protein/lipid interactions may also be induced by some agonists but not by others. Interactions between proteins and lipids are known to be modified following alterations in cell shape (29), and may be relevant to the inability of agonists to form low-affinity receptors on intact cells which have been suspended. A major difficulty in determining the nature of the form of the receptor with a low affinity for agonists is that we have not been able to produce this form of the receptor in membrane preparations by varying the reaction constituents. In addition, the rate of reversal of the low-affinity form of the receptor following removal of isoproterenol is very rapid (<1 min; ref. 23). This precludes the possibility of studying this phenomenon on membranes prepared from cells previously incubated with an agonist. One approach in trying to understand the basis of the low-affinity form of the receptor may be to determine other aspects of *beta*-adrenergic receptor/adenylate cyclase interactions that are differentially affected by prototype agonists such as isoproterenol and zinterol. We have recently observed that, following exposure of intact cells to isoproterenol but not to zinterol, there is a decrease in GTP-stimulated adenylate cyclase activity.[3] These phenomena may be related.

Although intact L6 cells that had been detached from the plates were viable, incubation of suspended cells with agonists did not result in the appearance of receptors with a low affinity for agonists. When suspended cells were added to culture dishes treated with bovine serum albumin, some cells attached but the majority remained in suspension. Using this system, it was possible to study receptors on cells in suspension and in monolayer culture in the same wells under identical conditions. The data indicated that, once cells attached, the affinity of their receptors for agonists was markedly reduced following exposure to isoproterenol. On the other hand, the affinity of receptors on cells in suspension in the same wells was unaffected by the presence of isoproterenol. The inability of receptors on cells in suspension to form the low-affinity state did not appear to result from the loss in cytoskeletal organization because treatment of monolayer cells with colchicine or cytochalasin B did not affect the ability of isoproterenol to induce the low-affinity form of the receptor (Table 4).

It is possible that the agents used to detach cells were also producing nonspecific effects or possibly removing a component of the cell necessary for the formation or maintenance of the low-affinity form of the receptor. The fact that EDTA does not release cell surface components (30) suggests that this is unlikely. These issues were addressed experimentally by using two different agents which detach cells in different ways (30–32), by performing assays on intact cells in monolayer cultures following brief exposure to EDTA or trypsin, by assaying suspended and newly attached cells in the same wells under identical conditions, by including divalent cations and soybean trypsin inhibitor in binding assays with suspended cells, and by performing binding assays on membrane preparations in the presence of concentrations of EDTA or trypsin used to suspend cells. The results of all of these experiments suggest that the differences in receptors on attached and suspended cells did not result from exposing cells to EDTA or trypsin but rather reflect the differences in the physical states of attached versus suspended cells. Although the form of the receptor with a low affinity for agonists was observed only in studies of attached cells, contact with the substratum was not sufficient by itself to allow formation of this form of the receptor because cells killed by freezing remained attached to the dish but had receptors with a high affinity for isoproterenol (Table 4).

The data presented in these studies suggest that initially *beta*-adrenergic receptors on intact cells in monolayer culture have the same properties as receptors on membranes when studied in the presence of GTP. Certain agonists such as isoproterenol, Cc-25, fenoterol, epinephrine, and norepinephrine can rapidly convert the receptors to a form with a low affinity for agonists. Other agonists, such as zinterol, salmefamol, dobutamine, and ephedrine, do not appear to be capable of converting receptors to another form and therefore interact in a similar way with receptors on both broken cells and intact cells in monolayer culture. Some drugs, including terbutaline, metaproterenol, OPC 2009, soterenol, salbutamol, carbuterol, and sulofonterol, convert some but not all of the receptors on intact cells to a form with a low affinity for agonists. Therefore, following exposure of attached cells to these drugs, two forms of the receptor exist at the same time. It appears that the form of the receptor with a low affinity for agonists is either not formed or is formed but not stabilized on cells in suspension.

[3] R. N. Pittman, R. A. Rabin, and P. B. Molinoff, in preparation.

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