MOLECULAR PHARMACOLOGY, 14, 549-561

Radioligand Binding to *Beta* Adrenergic Receptors of Intact Cultured S49 Cells

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(Received December 19, 1977)
(Accepted March 17, 1978)

SUMMARY

INSEL, PAUL A. & STOOLMAN, LLOYD M. (1978) Radioligand binding to *beta* adrenergic receptors of intact cultured S49 cells. *Mol. Pharmacol.*, 14, 549-561.

Using the radioligand [^{125}I]iodohydroxybenzylpindolol ([^{125}I]IHYP), we have characterized the *beta* adrenergic receptors of intact S49 lymphoma cells. Washed cells are incubated with the radioligand in a physiological growth medium containing 1 mM ascorbic acid and 0.1% horse serum. Binding of [^{125}I]IHYP saturates with time, equilibrium being achieved in 45-60 min, and is linear with cell number. Scatchard analysis of binding at equilibrium indicates one class of binding sites with a K_d equal to 28 pM and 1200-1300 binding sites/cell; this number of binding sites is about 4 times higher than previously observed with disrupted S49 cells. Independent estimates of the K_d derived from the forward and reverse reaction rates of binding are in agreement with the K_d derived from equilibrium studies. Competition for [^{125}I]IHYP binding sites by *beta* adrenergic antagonists corresponds closely to estimates of potency of the same antagonists in blockade of *beta* adrenergic-stimulated cyclic 3',5'-AMP accumulation. In contrast, *beta* adrenergic agonists are much more potent in stimulating cyclic AMP accumulation than in competing with [^{125}I]IHYP for *beta* adrenergic binding sites on intact S49 cells. Furthermore, agonists appear more potent in competing for [^{125}I]IHYP binding sites of disrupted than of intact S49 cells. The lower potency of *beta* adrenergic agonists in binding studies of intact cells may be attributable to the presence of endogenous guanyl nucleotides and, perhaps, to the more efficient coupling between receptors and adenylate cyclase of the intact cells. That this lower potency of agonists is not an artifact of studies with [^{125}I]IHYP is indicated by similar findings with the radioligand [^{3}H]dihydroalprenolol ([^{3}H]DHA). [^{3}H]DHA binds to intact S49 cells rapidly (reaching equilibrium in less than 3 min) and reversibly, with a K_d of 0.67 ± 0.08 nM. An identical number of binding sites is found on intact S49 cells whether [^{3}H]DHA or [^{125}I]IHYP is used, and studies in which adrenergic agents compete for ligand binding sites yield similar results for both radioligands. These studies of binding to *beta* adrenergic receptors of intact cultured cells using either [^{125}I]IHYP or [^{3}H]DHA offer a useful tool that should complement and extend studies of receptors on plasma membranes isolated from such cells.

INTRODUCTION

Studies of plasma membrane receptors for catecholamines and other membrane-

This work was supported by Grants GM 16496, GM 00001, and HL 06285 from the National Institutes of Health.

[1] Established Investigator of the American Heart Association.

[2] Recipient of a medical student research fellowship from the Pharmaceutical Manufacturers of America and a medical student summer research fellowship from the University of California, San Francisco.

0026-895X/78/0144-0549$02.00/0

active hormones that stimulate adenylate cyclase [ATP pyrophosphatelyase (cyclizing), EC 4.6.1.1] have predominantly been investigations of crude and purified membranes prepared from target tissues. Attempts to correlate hormonal binding with activation of cyclase have suffered from several methodological difficulties, including use of heterogeneous tissues as sources for membranes, measurement of binding and cyclase under different assay conditions, and lack of knowledge regarding the effect of cellular disruption on the hormone-stimulated cyclase systems (1–5). This last difficulty is perhaps the most vexing to consider, because it has generally been necessary to break cells in order to study hormonal binding together with activation of cyclase. As a result, most investigators have chosen to ignore the commonly observed result that breaking cells and preparing membranes produces a marked (more than 70%) loss in hormone-sensitive cyclase activity (5–7). In several systems, compounds such as purine nucleotides, in particular GTP or one of its nonhydrolyzable analogues [e.g., Gpp(NH)p],[3] must be added to plasma membranes in order to observe hormonal activation of adenylate cyclase (8–11). Thus, in order to examine whether data derived from such experiments in membrane preparations are applicable to the physiological conditions of intact cells, we have studied *beta* adrenergic receptors in unbroken, viable cells.

We chose the S49 lymphoma cell, a model system for studying catecholamine action (4, 12), as a source of homogeneous cells, and we compared cAMP accumulation and binding to receptors under similar experimental conditions. Because of the low numbers of receptors estimated from similar studies with broken S49 cells (13), we chose the radioligand [^{125}I]iodohydroxybenzylpindolol (14–17) for this assay.

In defining binding of a radioligand to *beta* adrenergic receptors, several criteria (18) must be fulfilled: (a) saturation of binding with time and ligand concentration, (b) stereoselectivity [(−) isomers more potent than (+) isomers] in competition for binding sites by agonists and antagonists, (c) rank order of potency for adrenergic agonists that corresponds to the biological activity of these agents, and (d) reversibility of binding. These criteria have been fulfilled for binding of [^{125}I]IHYP to intact S49 cells. One discrepancy in these results is the lower affinity of *beta* adrenergic agonists in binding studies than in assays of cAMP accumulation. Similar studies using the radioligand [^{3}H]dihydroalprenolol indicate comparable estimates for the number of *beta* adrenergic binding sites on intact S49 cells and for the affinity of adrenergic agonists with these binding sites.

[3] The abbreviations used are: Gpp(NH)p, 5′-guanylylimidodiphosphate; cAMP, cyclic adenosine 3′,5′-monophosphate; [^{125}I]IHYP, [^{125}I]iodohydroxybenzylpindolol; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; Ro 20-1724, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone; [^{3}H]DHA, [^{3}H]dihydroalprenolol.

METHODS

Cell preparation. Wild-type S49.1 mouse lymphoma cells (19) were propagated in suspension culture as previously described (12) in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum and 3 g of glucose per liter (final concentration). Cells that were growing logarithmically in either spinner flasks or individual T-flasks at a density of 0.5–2.5 × 10^6 cells/ml were routinely prepared as follows. Cells were counted in a hemocytometer after staining with trypan blue, and aliquots of cells in which the viability (exclusion of trypan blue) was greater than 80% were then centrifuged at 65 × *g* for 5 min in a refrigerated centrifuge. The cellular pellet was resuspended in Dulbecco's modified Eagle's medium minus $NaHCO_3$ and supplemented with 0.1% heat-inactivated horse serum, 20 mM HEPES (Na^+), pH 7.4, and 1 mM ascorbic acid (termed assay buffer), centrifuged at 65 × *g* for 5 min, and then resuspended in assay buffer. This wash procedure and assay buffer were chosen after several alternative conditions (different buffers, pH values, absence of horse serum) were found to decrease the number of viable cells during both the initial wash procedure and subsequent incubations for several hours at 37°. Using the method described, cell viability remained greater than 80% and total cell numbers were unchanged during prolonged incuba-

tions. Purified plasma membranes from these cells were prepared as previously described (20).

[^{125}I]Iodohydroxybenzylpindolol binding assay. Cells at a density of 1–2 × 10^6/ml were incubated at 37° in 3 ml of assay buffer containing the *beta* adrenergic antagonist [^{125}I]iodohydroxybenzylpindolol and various additions. [Although hydroxybenzylpindolol has been reported to have agonist activity in adipocytes (21), we find no such activity in S49 cells.] Binding reactions were terminated by adding a 0.5-ml aliquot of incubation mixture to 2.5 ml of a 37° solution containing 5 mM potassium phosphate (pH 7.0), 1 mM $MgSO_4$, and 0.01 mM (±)-propranolol and overlaying a glass fiber filter (Gelman A/E). The samples were filtered at a speed of 50–60 ml/min by applying vacuum, and the filters were then washed with 30 ml of the same buffer used to terminate the reactions except that it lacked propranolol. Filters were dried by increasing the vacuum and then were counted in a liquid scintillation system (efficiency, 60%). All samples were run in duplicate to quadruplicate. Duplicate determinations generally agreed within 10%. Aliquots of cells were maintained on ice prior to addition to the other reagents, which had been warmed to 37°. In binding experiments performed for less than 30 min, cells were incubated to 37° for at least 5 min prior to addition to other reagents.

cAMP accumulation assays. Unless otherwise indicated, aliquots of cells at 1–2 × 10^6/ml (final concentration) were incubated in assay buffer containing 0.1 mM Ro 20-1724, a potent inhibitor of cyclic nucleotide phosphodiesterase in S49 cells (22), and various additions. Rates of accumulation of cAMP in cells were nonlinear, being most rapid at the earliest time chosen. For convenience, samples were incubated for 10–15 min at 37°, although estimates of K_a values for agonists are similar to those reported here if earlier times are chosen. Reactions were terminated by adding 4 ml of ice-cold assay buffer containing 0.1 mM (±)-propranolol and 0.1 mM Ro 20-1724, centrifuging the sample, and separating the cells from the medium. The cellular pellets were then placed in a boiling water bath for 5 min, and the cAMP content in the buffer was assayed using a competitive binding protein method (22). In other experiments, total (cells plus medium) cAMP levels were determined by stopping reactions with 5% trichloracetic acid (the two different methods of stopping the reactions yield comparable estimates for cAMP accumulation when cells are incubated less than 15 min) and separating cAMP with anion-exchange columns (Biorad AG1-X, formate form, 200–400 mesh). These columns retain [^{125}I]IHYP under the conditions used to elute cAMP (23). [^{3}H]cAMP was added to the samples placed on the columns in order to correct for recovery of cAMP (usually 60–80%). After lyophilization of the column eluate, samples were resuspended and cAMP was assayed as noted above.

[^{3}H]Dihydroalprenolol binding assay. Cells at a density of 2–2.5 × 10^7/ml were incubated at 37° for 5 min in 0.25 ml of assay buffer (which sometimes contained 0.1% bovine serum albumin in lieu of 0.1% horse serum; this substitution had no effect on results), the *beta* adrenergic antagonist [^{3}H]dihydroalprenolol (12,000–1,000,000 cpm/ml), and various additions. Binding reactions were terminated by adding 3 ml of the same stopping buffer as was used in the [^{125}I]IHYP assay, but for the [^{3}H]DHA assay this solution was maintained on ice. The samples were immediately filtered over glass fiber filters (Gelman A/E) and rinsed with 15 ml of ice-cold stopping buffer that lacked propranolol. Propranolol was included in the stopping solution of the two binding assays in order to eliminate nonspecific retention of counts on the filters and thus to equalize samples incubated with ligand alone or radioligand plus competitors of binding. The dried filters were counted in a liquid scintillation system. All samples were run in duplicate and generally differed from each other by less than 10%. Nonspecific binding was determined by incubating samples in the presence of 0.3 μM (−)-alprenolol, a concentration two to three orders of magnitude greater than K_i for this compound. Nonspecific binding was less than 25% of total binding at concentrations of [^{3}H]DHA less than 5 nM. Specific binding, the difference between total and nonspecific binding, was linear at cell densities from 0.4 to 4 × 10^7/ml and reached equilib-

rium in less than 3 min at 37°.

Materials. All chemicals were obtained from standard sources except as follows. [^{125}I]IHYP was prepared as described previously (16) approximately every 4–6 weeks. Purity of the product was routinely verified on thin-layer chromatograms (16). Hydroxybenzylpindolol was a gift from Dr. D. Hauser (Sandoz), Ro 20-1724 was a gift from Dr. H. Sheppard (Roche), and hydroxybenzylisoproterenol was provided by Dr. R. J. Lefkowitz. Other agents were obtained as follows: (−)-propranolol and (+)-propranolol, from Ayerst; pindolol, from Sandoz; and (−)-isoproterenol, (+)-isoproterenol, (−)-epinephrine, and (−)-norepinephrine, from Sterling-Winthrop. [^{3}H]DHA (48 Ci/mmole) was obtained from New England Nuclear.

Calculations. K_i values for antagonism of bound [^{125}I]IHYP by *beta* adrenergic agents were calculated from the formula $K_i = K_d/(S + K_d) \times I_{50}$ (24), where K_d is the dissociation constant for [^{125}I]IHYP, S is the concentration of [^{125}I]IHYP in a given experiment, and I_{50} is the concentration of agent inhibiting specific binding 50%. In cAMP synthesis assays, K_a values for agonists were the concentrations at which accumulation of cAMP was 50% of its maximum; K_i values in cAMP synthesis assays for antagonists were calculated from the formula above, substituting K_a for K_d and letting I_{50} represent the concentration of antagonist inhibiting the accumulation of cAMP 50%.

RESULTS

Nonspecific binding of [^{125}I]IHYP and separation of bound from free ligand. Because of the availability of only small quantities of hydroxybenzylpindolol and iodohydroxybenzylpindolol, we, as well as others (16, 20), chose the *beta* adrenergic blocking agent (−)-propranolol for estimating nonspecific binding of [^{125}I]IHYP. (−)-Propranolol competes for [^{125}I]IHYP binding sites at two classes of sites: a higher-affinity, stereoselective site that yields an I_{50} value of 3 nM, and a lower-affinity site that is not stereoselective (Fig. 1). In view of the importance of stereoselectivity for activity of *beta* adrenergic agents (4, 18, 25), we chose a (−)-propranolol concentration of 1.0 μM to define nonspecific binding. Specific binding therefore is the difference between counts bound in the absence and presence of 1.0 μM (−)-propranolol. At concentrations of 40–50 pM [^{125}I]IHYP and 1–2 × 10^6 cells/ml, nonspecific binding is 31% ± 5% (mean ± SD) of total counts bound, in contrast with results of binding studies of crude particulates, in which nonspecific binding is less than 20% of total binding (13), and in purified plasma membranes, in which nonspecific binding is less than 5% of the total counts bound (20). The conditions chosen to separate bound from free ligand were designed to maximize the ratio of specific to nonspecific binding (data not shown). Under these conditions specific binding is linear with cell concentrations between 0.4 and 5.0 × 10^6 cells/ml (data not shown).

Kinetics of binding and reversal of bound counts. Specific binding achieved equilibrium over a 45–60-min period (Fig. 2). This time course, which parallels the

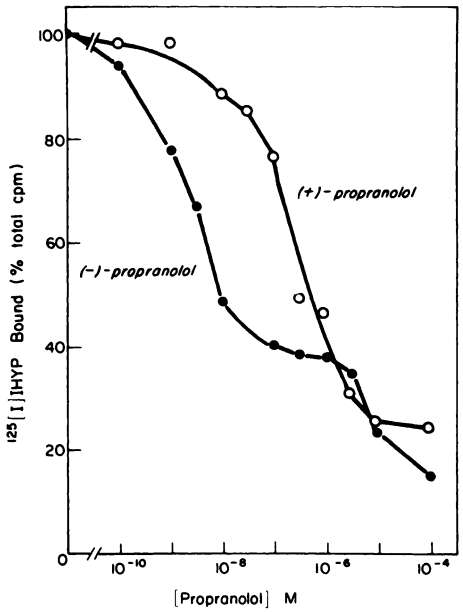

FIG. 1. *Competition for [^{125}I]IHYP binding sites by (−)- and (+)-propranolol*

Intact cells were incubated with [^{125}I]IHYP (50 pM) and the indicated concentrations of (−)- and (+)-propranolol for 60 min. Binding was determined as described in METHODS. Results plotted are values derived from duplicate determinations in at least three experiments.

onset of *beta* adrenergic blockade by [^{125}I]IHYP in these cells (Fig. 2), yields a rate of association (k_1) of 9.0×10^8 M^{-1} min^{-1}. The concentration of [^{125}I]IHYP used in the experiment depicted in Fig. 2 (100 pM) is much greater than the concentration of binding sites in the assay (16 pM), and thus the forward reaction rate can be considered a pseudo-first-order reaction that depends on the concentration of receptors (26).

A 60-min incubation was routinely used for binding experiments. Bound counts extracted from cells incubated with [^{125}I]-IHYP for 60 min migrated on thin-layer chromatography with [^{125}I]IHYP, and [^{125}I]IHYP that was incubated with cells for 60 min could be used in binding studies with fresh cells and was bound to the same extent as was fresh [^{125}I]IHYP.

Reversal of [^{125}I]IHYP binding by adding 1.0 μM (−)-propranolol indicates that specific binding reverses with a $t_{1/2}$ of approximately 100 min (k_2 = 0.0069 min^{-1}) (Fig. 3), a value similar to previous estimates of the off rate in broken S49 cell preparations (13, 20).

A small component (10–15%) of bound ligand dissociates at a faster rate than does the majority. This is similar to previous findings with reversal of binding from particulate fractions prepared from S49 (13)

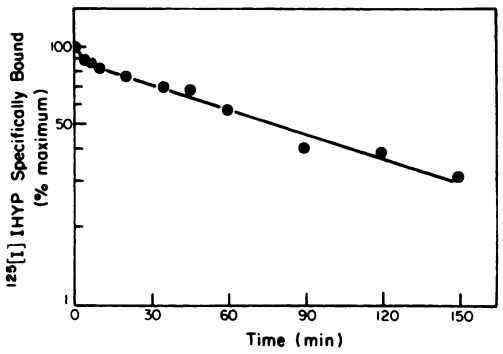

FIG. 3. *Reversal of binding of [^{125}I]IHYP by 1.0 μM (−)-propranol*

Cells (2×10^6 cells/ml) were incubated with [^{125}I]IHYP (53 pM) for 60 min. (−)-Propranolol was then added to a final concentration of 1.0 μM, and specifically bound counts, as defined in the legend to Fig. 2, were determined at the indicated times in duplicate aliquots. Data are expressed as percentage of counts specifically bound at 60 min.

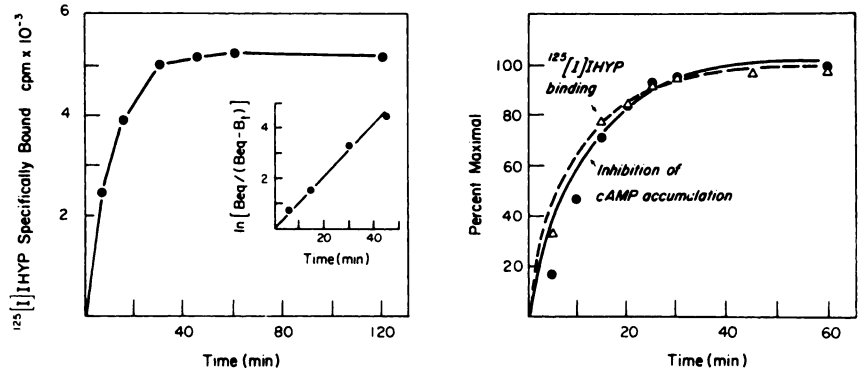

FIG. 2. *Time course of binding of [^{125}I]IHYP (left and right panels) and inhibition of cAMP accumulation by [^{125}I]IHYP (right panel) in intact S49 cells*

In the experiment shown at left, cells (2×10^6/ml) were incubated with [^{125}I]IHYP (100 pM) in the absence and presence of 1 μM (−)-propranolol. Specific binding, i.e., the difference between counts bound in the absence and presence of 1 μM (−)-propranolol, was determined in duplicate aliquots at the indicated times. The inset plots the regression line (r = 0.99), determined by least-squares fit of ln $B_{eq}/(B_{eq}-B_t)$ at different times, where B_{eq} = binding at equilibrium and B_t = binding at time t. k_{ob}, the slope of this line, can be used to calculate the second-order rate constant k_1 from the equation $k_1 = (k_{ob} - k_2)/$[^{125}I]IHYP, where k_2 is the rate constant for the reverse (dissociation) reaction (Fig. 3) and [^{125}I]IHYP is the concentration of radioligand in the experiment (100 pM). In the experiment shown at right, cells (1.5×10^6/ml) and 60 pM [^{125}I]IHYP were incubated in assay buffer at 37°. At the indicated times specific binding of [^{125}I]IHYP and cAMP accumulation that could be stimulated by 0.1 mM (−)-isoproterenol and 0.1 mM Ro 20-1724 during a 15-min period were determined. Total cAMP levels were determined as described in METHODS. Each point is the average of duplicate determination. Data are expressed as percentage of maximal *beta* adrenergic blockade or [^{125}I]IHYP specifically bound.

and other (16) cultured cells. If bound counts are dissociated with 100 μM (−)-propranolol, this component of rapid ($t_{1/2}$ = 5 min) reversal is more prominent (approximately 30% bound counts) (Fig. 4). When binding is studied in the presence of 1.0 μM pindolol and then 100 μM (−)-propranolol is added (inset, Fig. 4), only the rapid phase of reversal is noted ($t_{1/2}$ = 3 min). This suggests that the rapid component dissociated by 100 μM (−)-propranolol represents that bound to the lower-affinity, nonstereoselective (Fig. 1) site.

Equilibrium binding of [^{125}I]IHYP. Binding of increasing concentrations of [^{125}I]IHYP with intact S49 cells is saturable, and Scatchard analysis (27) indicates a single class of binding sites (Fig. 5). The K_d of binding is 28 ± 2 M (mean ± SE, n = 6), and the number of binding sites per cell is 1270 ± 140. This measured K_d is similar to the value estimated from the ratio of the association and dissociation rate constants (8 pM). The lower-affinity sites noted in the reversal experiments are not detected in the equilibrium studies.

Competition for [^{125}I]IHYP binding sites and inhibition of cAMP stimulation by adrenergic antagonists. Beta adrenergic receptors typically exhibit stereoselectivity in the expression of pharmacological activity. As Fig. 1 shows, (−)-propranolol is approximately 100 times more potent in in-

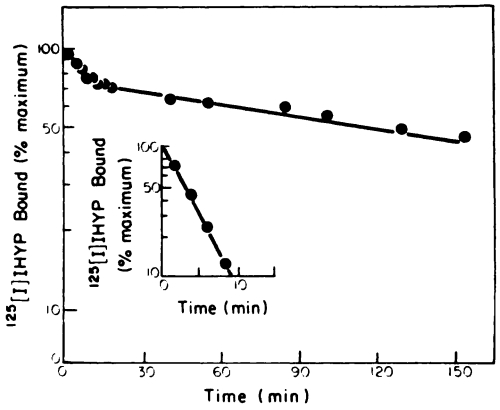

FIG. 4. *Reversal of binding of [^{125}I]IHYP by 0.1 mM (−)-propranolol*

Cells (3 × 10^6 cells/ml) were incubated with [^{125}I]IHYP (100 pM) for 60 min. (−)-Propranolol was then added to a final concentration of 0.1 mM, and bound counts were measured as described in METHODS. The inset shows the reversal of binding of [^{125}I]IHYP to cells in the presence of 1 μM pindolol by increasing the propranolol concentration to 0.1 mM after 60 min. Data are expressed as percentage of counts bound at 60 min.

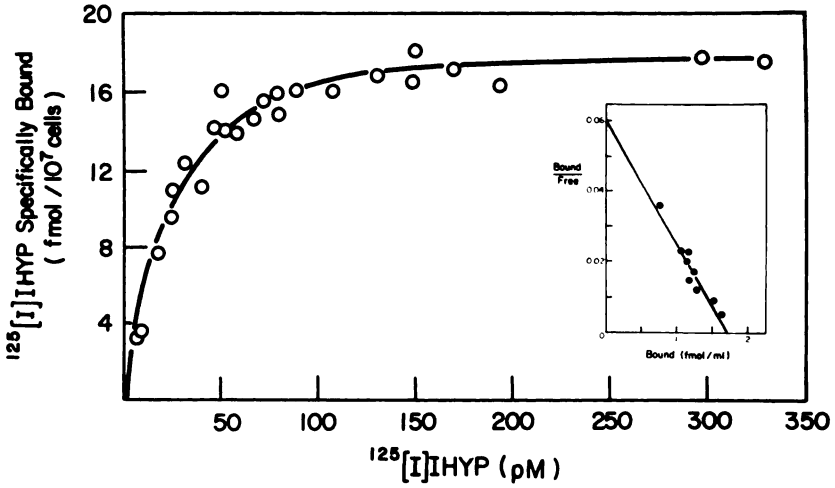

FIG. 5. *Specific binding as a function of concentration of [^{125}I]IHYP*

Cells and various concentrations of [^{125}I]IHYP were incubated in assay buffer for 60 min. Total, nonspecific, and specific binding was determined at each concentration of [^{125}I]IHYP. The data shown are derived from three separate experiments, and each point is the average of duplicate determinations. The inset shows a Scatchard plot of [^{125}I]IHYP binding to intact S49 cells in one experiment in which the cell concentration was 0.8 × 10^6/ml. The line is a regression line (r = 0.95), determined by least-squares fit, that indicates a K_d of 30 pM and a number of binding sites equivalent to 1290 sites/cell.

hibiting binding of [^{125}I]IHYP than is (+)-propranolol. Other adrenergic antagonists (Fig. 6) show a close correspondence between ability to inhibit [^{125}I]IHYP binding and isoproterenol-stimulated cAMP accumulation in S49 cells. The ratios of K_i values derived in assays of [^{125}I]IHYP binding and of inhibition of cAMP accumulation are 1-3 (Table 1). Other compounds (at concentrations of 0.1 mM), including dihydroxyphenylalanine, catechol, phenoxybenzamine, ascorbic acid, and phentolamine, do not inhibit [^{125}I]IHYP binding to intact S49 cells.

Competition of [^{125}I]IHYP binding sites and stimulation of cAMP synthesis by beta adrenergic agonists. Beta adrenergic agonists are also stereoselective in stimulating

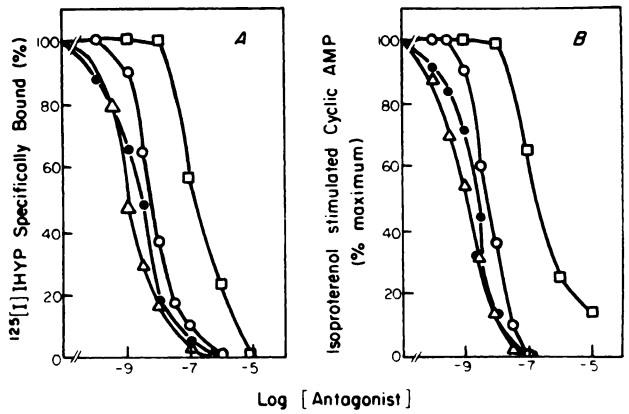

FIG. 6. *Competition for [^{125}I]IHYP binding sites (A) and inhibition of isoproterenol-stimulated cAMP accumulation (B) by beta adrenergic antagonists in intact S49 cells.*

A. Various concentrations of (−)-propranolol (●——●), (±)-pindolol (○——○), (±)-hydroxybenzylpindolol (△——△), and (±)-dichloroisoproterenol (□——□) were incubated with cells and [^{125}I]IHYP for 60 min. Specifically bound counts were then determined as described in the legend to Fig. 2. The data shown are means of two to four aliquots in at least two separate experiments for each compound.

B. Various concentrations of the same agents were incubated with cells and 0.1 μM (−)-isoproterenol for 15 min. Cellular accumulation of cAMP was determined as described in METHODS. The data are expressed as percentage of the amount of cAMP accumulation stimulated by isoproterenol in the absence of added antagonist.

TABLE 1

Apparent equilibrium constants for adrenergic agents in binding assays with [^{125}I]IHYP and [^{3}H]DHA and in cAMP accumulation assays in intact S49 cells

Competition for [^{125}I]IHYP and [^{3}H]DHA binding sites and measurement of cellular cAMP levels were performed as described in METHODS. Data are mean estimates from at least two experiments for each compound. Estimates for individual compounds varied between experiments by less than a factor of 3.

Addition	K_a or K_i from cAMP accumulation assay	K_i from [^{125}I]IHYP binding assay	K_i from [^{3}H]DHA binding assay
	nM	*nM*	*nM*
***Beta* adrenergic antagonists**			
(−)-Propranolol	0.26	0.71	0.44
(+)-Propranolol	32	29	28
(±)-Pindolol	0.49	1.1	
(±)-Hydroxybenzylpindolol	0.13	0.34	0.50
(±)-Dichloroisoproterenol	24	54	12
***Beta* adrenergic agonists**			
(±)-Hydroxybenzoylisoproterenol	1.5	310	120
(−)-Isoproterenol	12	3,800	1,000
(+)-Isoproterenol	780	31,000	26,000
(−)-Epinephrine	37	23,000	24,000
(−)-Norepinephrine	4,300	72,000	72,000

cAMP accumulation and in competing for [^{125}I]IHYP binding sites (Fig. 7 and Table 1). The rank order of potency for these two processes is identical for the compounds tested [hydroxybenzylisoproterenol > (−)-isoproterenol > (−)-epinephrine > (+)-isoproterenol > (−)-norepinephrine]. In contrast with the results observed with *beta* adrenergic antagonists, however, the absolute K_i values for the stimulation of cAMP accumulation by the agonists (Table 1) are about two orders of magnitude lower than are the comparable K_i values for inhibition of [^{125}I]IHYP binding, and the resultant K_i/K_a ratios range from 20 to 600.

We tested whether this discrepancy in K_i/K_a ratios resulted from artifacts in our assay system. We find that catecholamines, [^{125}I]IHYP, and receptors are not extensively degraded during the binding assay and that, in addition, bound counts do not appear to represent intracellular accumulation of [^{125}I]IHYP. Catecholamine degradation does not occur, because the pharmacological potency of isoproterenol does not decrease after an incubation for 120 min under these assay conditions (including cells). Similarly, isoproterenol-stimulated cAMP accumulation is equivalent in cells previously incubated (in the absence of agonist) for 5–10 min at 37° and in cells incubated for over 3 hr. Degradation of [^{125}I]IHYP is unlikely, because bound radioligand, extracted from cells with organic solvent, chromatographs as [^{125}I]IHYP, and, in addition, [^{125}I]IHYP that has been incubated with cells for 60 min is bound to the same extent as is fresh [^{125}I]IHYP.

The nonspecific transport of [^{125}I]IHYP into cells seems unlikely in view of the following: (a) the time course of [^{125}I]IHYP binding and the institution of pharmacological blockade by this antagonist are virtually identical (Fig. 2), which suggests that binding occurs at a pharmacologically active site and not at a separate transport site; and (b) the striking K_i/K_a discrepancy exists only for agonists; one would expect that if transport of [^{125}I]IHYP occurred at a site not involved in the catecholamine response of the cell, altered potencies might be observed for both agonists and antagonists, rather than just for agonists.

A possible explanation for the K_i/K_a discrepancy for *beta* adrenergic agonists would be spare receptors; i.e., only a fraction of the available receptors for the agonists needs to be occupied to produce a maximal biological response. A way to test this would be to block irreversibly a portion of the receptors and to ask whether the cells are then capable of generating a maximal

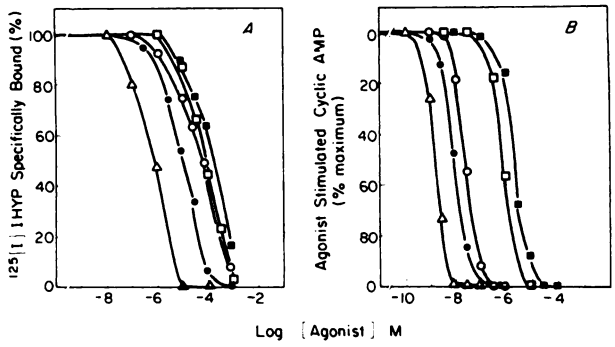

FIG. 7. *Competition for [^{125}I]IHYP binding sites (A) and stimulation of cAMP synthesis (B) by beta adrenergic agonists in intact S49 cells*

A. Various concentrations of (±)-hydroxybenzylisoproterenol (△——△), (+)-isoproterenol (□——□), (−)-isoproterenol (●——●), (−)-epinephrine (○——○), and (−)-norepinephrine (■——■) were incubated with cells and [^{125}I]IHYP (50 pM) for 60 min. Specifically bound counts were then determined as described in the legend to Fig. 2. The data shown are means of at least two aliquots for each compound.

B. Various concentrations of the same agents were incubated with cells for 15 min. Cellular accumulation of cAMP was assayed as described in METHODS. The data are expressed as percentage of maximal response to the individual compounds and are means of at least two determinations. Each compound at maximal doses produced equivalent stimulation of cAMP synthesis.

response to a *beta* adrenergic agonist. The slow dissociation rate of [^{125}I]IHYP (100 min) facilitated our using the radioligand as such a blocking agent of *beta* adrenergic receptors in S49 cells (Table 2). Preliminary incubation of cells with 30 pM [^{125}I]IHYP, which should occupy approximately 50% of the *beta* adrenergic receptors (Kd = 28 pM), reduces isoproterenol-stimulated cAMP accumulation by an equivalent amount. Similarly, occupancy of 95% of the receptors with [^{125}I]IHYP results in a greater than 90% decrease in isoproterenol-stimulated cAMP accumulation. Thus spare receptors do not appear to explain the K_i/K_a discrepancy of S49 cells.

Guanyl nucleotides have several well-known effects on hormonal binding and stimulation of adenylate cyclase studied in membrane preparations (9–11, 20, 28–33). Because such effects are observed with *beta* adrenergic agonists but not with antagonists (20, 31, 32), nucleotides have been implicated as coupling agents between *beta* adrenergic receptors and adenylate cyclase. Addition of guanyl nucleotides to plasma membrane preparations of S49 cells decreases the potency of (−)-isoproterenol in competing for [^{125}I]IHYP binding sites on these membranes (20); in the absence of such nucleotides the potency of agonists in competing for [^{125}I]IHYP sites is one to two orders of magnitude greater than the values observed in intact S49 cells. Addition of 100 μM GTP or Gpp(NH)p is without effect on the isoproterenol competition for [^{125}I]-IHYP binding sites on intact S49 cells (data not shown). The lack of effect on intact cells of such highly polar compounds might simply be explained by the inaccessibility of nucleotides to regulatory sites within the plasma membrane. However, we find that the affinity of (−)-isoproterenol for [^{125}I]-IHYP binding sites in the intact cell is only about 3 times less than values determined in membrane preparations to which nucleotides have been added. Thus addition of nucleotides to the membranes diminishes the difference in binding of agonists between whole cells and membranes. Furthermore, competition with [^{125}I]IHYP by (−)-isoproterenol indicates a Hill coefficient of 0.66 for plasma membranes examined in the absence of added nucleotides. We find, as did Ross *et al.* (20), that this coefficient increases to values of 0.81 and 0.86 in the presence of 100 μM Gpp(NH)p and GTP, respectively. The Hill coefficient for whole cells examined in the absence or presence of nucleotides is 1.08. Thus, by this additional criterion, binding to *beta* adrenergic receptors of intact S49 cells resembles that of plasma membranes to which guanyl nucleotides are added.

TABLE 2

Effect of previous incubation of S49 cells with [^{125}I]IHYP on cellular response to isoproterenol

Wild-type S49 cells (1.5 × 10^6 cells/ml) were first incubated at 37° in medium in the absence and presence of [^{125}I]IHYP (30 or 190 pM) for 90 min. Aliquots were removed, and total (medium plus cellular) cAMP accumulation was determined during a second 15-min accumulation after adding 0.1 mM Ro 20-1724 and 0.1 mM (−)-isoproterenol. Incubations were terminated with trichloracetic acid and were assayed as described in METHODS. Data are expressed as means ± standard deviations.

Addition in initial incubation	Addition in second incubation	cAMP
		pmoles/10^7 cells/15 min
Medium	Ro 20-1724	46 ± 28
Medium	Isoproterenol	1247 ± 148
[^{125}I]IHYP (30 pM)	Isoproterenol	697 ± 87
[^{125}I]IHYP (190 pM)	Isoproterenol	148 ± 13

Binding of [^{3}H]DHA to intact S49 cells. Since experiments on stimulation of cAMP accumulation by agonists and intact cells routinely involve 10–15-min incubations whereas equilibrium binding studies of [^{125}I]IHYP with intact cells require at least 45 min, it is possible that the difference in potency of agonists in the two types of studies results from these differences in duration of the incubations. To test this possibility, we used another radioligand, [^{3}H]DHA, and found that intact S49 cells bind this ligand rapidly (equilibrium being achieved in less than 3 min) and reversibly (data not shown; $t_{1/2}$ = 5 min). Scatchard analysis of equilibrium binding studies (Fig. 8) indicates a K_d for [^{3}H]DHA of 0.67 ± 0.08 nM (mean ± SD) and 1270 ± 210 binding sites/cell. In competition experiments estimated K_i values (Table 1) for antagonists and agonists are similar to values noted with [^{125}I]IHYP.

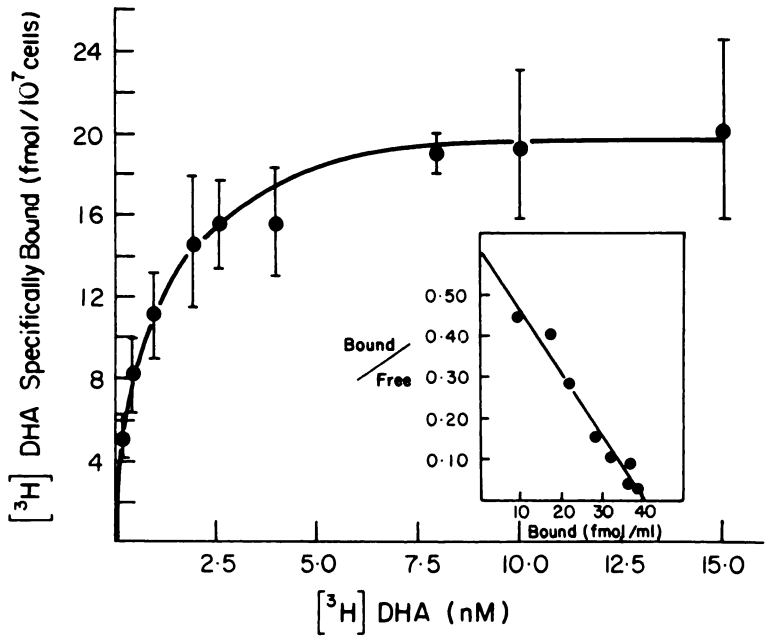

FIG. 8. *Specific binding as a function of concentration of [^{3}H]DHA*

Cells and various concentrations of [^{3}H]DHA were incubated in assay buffer (containing 0.1% bovine serum albumin in lieu of 0.1% horse serum) for 10 min. Total, nonspecific, and specific binding was determined at each concentration of [^{3}H]DHA. The data shown are means ± standard deviations of duplicate determinations derived from four to six separate experiments. The inset shows a Scatchard plot of [^{3}H]DHA binding to intact S49 cells in one experiment in which the cell concentration was 0.6×10^7/ml. The line is a regression line, determined by least-squares fit, that indicates a K_d of 0.63 nM and a number of binding sites equivalent to 1000 receptors/cell.

DISCUSSION

Binding of radioligands to putative *beta* adrenergic receptors in particulate preparations has been described in several tissues, including erythrocytes, lymphocytes, adipocytes, brain, liver, heart, uterus, and numerous tissue culture cells (for review, see ref. 4, 18). Although binding to *beta* adrenergic receptors in intact erythrocytes (34, 35) and to disrupted parathyroid cells (36) has recently been reported, this is the first study to examine in detail the properties of *beta* adrenergic binding to intact tissue culture cells under physiological conditions and, to our knowledge, the first study by one laboratory comparing results with two radioligands in the same tissue. Several preliminary reports of binding to intact HeLa cells (37), 3T3 cells (38), and L6 cells (39) have not indicated that the criteria (see INTRODUCTION) for binding to *beta* adrenergic receptors have been fulfilled.

In comparing the results of binding to *beta* adrenergic receptors in intact and broken S49 cells (13, 20), we find several similarities: kinetics of binding and dissociation constant for [^{125}I]IHYP, competition for [^{125}I]IHYP binding sites by *beta* adrenergic antagonists, and K_a values of agonists for stimulation of cAMP accumulation and adenylate cyclase. However, certain features distinguish between results in intact and disrupted S49 cells. We have previously reported (13) only 250–300 sites/cell in studies of broken cells but find that intact cells have 1200–1300 sites/cell. This difference may result from loss or inactivation of binding sites during preparation of S49 particulates. In view of the several similarities between the binding sites in intact and disrupted cells, the close parallelism between the time course of binding and *beta* adrenergic blockade by [^{125}I]IHYP, and the similar number of sites found with [^{3}H]DHA, the increased number of binding sites de-

tected in intact cells probably represent additional sites that are qualitatively similar to the sites in particulate preparations. A parallel observation compatible with this interpretation is the loss of adenylate cyclase activity after breaking cells and fractionating subcellular particles. Disruption of S49 cells [and of numerous other cells (1, 2, 5)] decreases the response of adenylate cyclase to isoproterenol and other hormones. We calculate that only 5–10% of the isoproterenol-stimulated cyclase activity, but 20–25% of the *beta* adrenergic receptors, is recoverable after cellular disruption and fractionation. Thus the amount of cAMP synthesized per agonist-receptor complex must be decreasing. Coupling between receptors and cyclase appears to be altered, therefore, when S49 cells are broken. Several investigators have suggested (8–11, 20, 31–33) that guanyl nucleotides are necessary components for full expression of hormonal activation of adenylate cyclase activity. The potency of agonists in competing with [^{125}I]IHYP binding sites of intact S49 cells is less than in studies of plasma membranes, and this difference decreases by addition of exogenous nucleotides to the membranes. This suggests that nucleotides are involved in coupling receptors to cyclase in intact cells (40).

In our studies of intact S49 cells, *beta* adrenergic agonists have a lower potency in competing with [^{125}I]IHYP or [^{3}H]DHA than in stimulating cAMP accumulation. This K_i/K_a discrepancy has not been observed in previously reported binding studies of intact frog (34) and turkey (34) erythrocytes, nor have we seen such differences in studies using crude particulate preparations of S49 cells (13). As noted above, the addition of nucleotides to purified plasma membranes of S49 cells changes the potency of agonists in radioligand binding and in adenylate cyclase assays. The result of this effect is to increase the K_i/K_a ratio to about 5 (20). Ross *et al.* (20) have suggested that this ratio may be a measure of the tightness of coupling between receptors and adenylate cyclase. In comparing several tissues, they found (4) that S49 cells were the most tightly coupled of all those that have been reported. Our results could indicate, then, that intact S49 cells are more tightly coupled than are plasma membranes prepared from these cells.

The existence of spare receptors—that is, larger numbers of receptors than are required for maximal pharmacological response—might explain the K_i/K_a discrepancy. However, two results indicate that spare receptors do not account for the observed discrepancy for agonists in binding and cAMP stimulation assays: (a) the studies described above, in which we used [^{125}I]IHYP as an essentially irreversible antagonist, and (b) a recently isolated variant S49 cell that has a decreased number of assayable *beta* adrenergic receptors with a commensurate decrease in isoproterenol-stimulated adenylate cyclase activity.[4]

One property of S49 cells that we have previously reported is the development of refractoriness, in which cellular response to *beta* adrenergic agonists decreases after a pronounced but transient stimulation of cAMP accumulation (22). We, as well as others (41), have found that membranes prepared from refractory cells display a decrease in maximal isoproterenol-stimulated adenylate cyclase activity and in the number of *beta* adrenergic receptors. Since studies with particulate preparations indicate that refractoriness is associated with a decrease in the number of *beta* adrenergic receptors without a change in affinity of these receptors for agonists or antagonists, the observed K_i/K_a discrepancy for agonists in intact cell binding studies appears to be a different phenomenon from refractoriness.

The reason for this discrepancy thus remains unexplained. One possible explanation would be that receptors exist in both agonist and antagonist configurations, as has been suggested for *alpha* adrenergic receptors in rat brain (42). Further studies using a radiolabeled *beta* adrenergic agonist (43) would be useful in testing this hypothesis.

In conclusion, [^{125}I]IHYP and [^{3}H]DHA appear to be useful ligands for probing *beta* adrenergic receptors on intact S49 cells. Binding studies with these ligands fulfill criteria required for interaction at such re-

[4] G. L. Johnson, H. R. Bourne, M. K. Gleason, P. Coffino, P. A. Insel, and K. L. Melmon, manuscript submitted for publication.

ceptors and thus provide assays that are faster and less traumatic to cells than are assays requiring cellular disruption and fractionation. The data suggest that differences between the effects of hormones in whole and broken cells reported in a variety of systems (5–11, 44, 45) may derive from changes in receptors due to the loss of endogenous nucleotides and, perhaps, other entities during cellular disruption and fractionation. Use of radioligands with homogeneous populations of viable, nondisrupted cells should assist in studies of interaction of receptors and adenylate cyclase and regulation of receptors by agonists (17, 22, 41) and other factors (37, 38, 46).

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Michele Sanda and Mary Gleason, the support of Dr. Kenneth Melmon, and the advice of Dr. Henry Bourne.

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