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Receptors for β-Adrenergic Agonists in Cultured Chick Ventricular Cells

Relationship between Agonist Binding and Physiologic Effect

JAMES D. MARSH AND THOMAS W. SMITH

The Cardiovascular Division, Brigham and Women's Hospital, and the Departments of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts 02115

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SUMMARY

To determine if the two-state, guanine nucleotide-modulated β-adrenergic receptor model elucidated in erythrocyte membranes accurately describes hormone binding to intact heart cells, and to determine the relationship of agonist binding to physiologic contractile response, we studied β-adrenergic antagonist and agonist binding to intact cultured heart cells and homogenates of these cells from embryonic chick ventricle and related the binding observations to alterations in amplitude of contraction of intact cells under identical conditions. The levo isomer of the β-adrenergic antagonist pindolol was radioiodinated, purified, and utilized to characterize the β-adrenergic receptor in intact, beating heart cells under physiologic conditions. Computer analysis of iodopindolol-binding isotherms revealed a $K_D = 22 \pm 3$ pM with $B_{max} = 10.3$ fmol/mg of protein in intact cells; in homogenates of cells, the K_D was 39 ± 12 pM in the absence of exogenous guanine nucleotides and 19 ± 7 pM in their presence. Estimation of the dissociation constant for iodopindolol binding to intact cells by kinetic methods yielded $K_D = 64$ pM. Binding was stereospecific, saturable, and identified a β_1-adrenergic receptor. Computer modeling of agonist competition curves indicated a single receptor state in intact cells with $K_D = 0.28$ μM for isoproterenol. However, in cell homogenates, two receptor states for agonists were identified with the high affinity state of the receptor having a $K_D = 3$ nM. Addition of guanine nucleotides to the cell homogenate reverted the receptor to a single state similar to that in intact cells. Under nonequilibrium binding conditions, a high affinity state for agonist was detected in intact cells with $IC_{50} = 1.1$ nM. The EC_{50} for isoproterenol-enhanced contractility was 6 nM and EC_{50} for cAMP response was 4 nM. At the isoproterenol concentration causing 50% maximal inotropic response, 67% occupancy of high affinity receptors occurs. Thus, there is a close relationship between high affinity receptor occupancy and augmentation of contractility in intact cells. These findings support the view that agonist interaction with the guanine nucleotide-sensitive, high affinity receptor state initiates the physiologic response of myocardial tissue to β-adrenergic agonists.

INTRODUCTION

Cultured myocardial cells constitute an experimental preparation of intense investigative interest. They are widely used for studies on cardiac development and innervation (1, 2), metabolism, contractile physiology (3), electrophysiology (4), and examination of pathophysiologic states such as ischemia and thyroid dysfunction (5, 6). This preparation also permits direct examination of the relation between hormone-receptor interaction and physiologic effect (3, 7, 8).

Contractile performance and metabolism of the heart are modulated by β-adrenergic agonists and antagonists. Thus, a detailed understanding of the function and regulation of the β receptor in intact cultured heart cells is of considerable interest, despite the fact that it is a more cumbersome experimental system, being dependent on primary cell cultures (3, 4, 9), than erythrocyte systems (10) or tumor cell lines (11, 12). Moreover, β-adrenergic receptor-mediated physiologic response may be probed in the cultured heart cell by a number of complementary techniques, including measurements of contractility (3), cAMP production, mono- and divalent cation fluxes and

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contents (13), and electrophysiologic analysis of single ion channels (14).

Previous studies of the relationship between β receptor occupancy and physiologic response in a variety of model systems (the response usually being cAMP accumulation or adenylate cyclase stimulation) have frequently demonstrated a major gap between K_D for agonist binding and EC_{50} for physiologic response (15). The hypothesized "spare" receptors are often numerically dominant. In the C62B rat glioma cell line, for example, it was calculated that occupancy of one receptor could fully activate adenylate cyclase in that cell (16). Should this be the case for the heart cell, it would have profound implications for receptor regulation and expression of the contractile response to neurotransmitters and pharmacologic agonists and antagonists (17). Recent work on identification of β receptors in intact tumor cells (11, 12, 19) under conditions where there is minimal agonist-induced receptor regulation reports the existence of a high affinity state for receptors and much less discrepancy between K_D and K_{act}.

Several laboratories have estimated agonist affinity for the β receptor in intact cultured heart cells or cell homogenates, with widely disparate results (7–9). The experimental systems used did not permit careful correlation between K_D and physiologic effect. From these experiments in other model systems, it is difficult to predict with any certainty the model of the β receptor most relevant to intact cardiac tissue. In systems as seemingly similar as frog and turkey erythrocytes or astrocytoma and glioma cell lines, the β receptor functions and desensitizes in importantly differing ways. Thus, in the frog erythrocyte, agonist occupancy of the receptor leads to formation of a transient ternary complex including a high affinity state of the receptor (10, 19, 20). With activation of adenylate cyclase, there is rapid dissociation of this complex, with production of a low affinity receptor state and subsequent receptor internalization (21). In turkey erythrocytes, the receptor is phosphorylated but not internalized (22). In C62B glioma cells [but not 1321N astrocytoma cells (18)], there are abundant spare receptors. In S49 lymphoma cells, receptor regulation does not require productive interaction with the guanine nucleotide regulatory protein [N_s (11)], nor does it require receptor phosphorylation. Therefore, as an approach to resolving uncertainties about the cardiac β receptor system, we examined antagonist and agonist binding to the β receptor of intact cultured heart cells and homogenates and directly related binding properties to alterations in cAMP content and contractile response under nearly identical physiologic conditions.

MATERIALS AND METHODS

Tissue culture. Monolayer cultures of beating chick embryo ventricular cells were prepared as previously described (3). Briefly, 10-day-old chick embryo hearts were removed under sterile conditions and the ventricles were cut into fragments and placed in Ca- and Mg-free Hanks' solution. The ventricular fragments were gently agitated in 10-ml of 0.025% trypsin in Ca- and Mg-free Hanks' solution at 37° for five cycles of 7 min each. The suspension containing dissociated cells was placed in 10 ml of cold medium containing 50% heat-inactivated fetal calf serum and 50% Ca- and Mg-free Hanks' solution. This suspension was centrifuged at 300 × *g* for 10 min, the supernatant phase was discarded, and the cells were resuspended in culture medium consisting of 6% heat-inactivated fetal calf serum, 40% medium 199, 0.1% penicillin-streptomycin antibiotic solution, and 54% low potassium salt solution. Final concentrations (in millimolar) in the culture medium were: Na, 144; K, 4.0; Ca, 0.97; HCO_3, 18; Mg, 0.8; and Cl, 131. The suspension of cells was diluted to 4 × 10^5 cells/ml and placed in plastic tissue culture dishes. Cultures were incubated in a humidified 5% CO_2-95% air atmosphere at 37°. Confluent monolayers in which at least 70% of the cells were contracting developed by 3 days in culture. In some areas of the culture, there were two to three layers of cells present. All studies were done at either day 3 or 4 in culture, pilot experiments having shown no difference between results on those days.

Preparation of iodopindolol. Iodopindolol was prepared by the method of Barovsky and Brooker (23). IPIN[1] prepared by this method was purified to an estimated specific activity of 2200 Ci/mmol. IPIN was continuously protected from light exposure and was stored at −20°. The specific activity of IPIN was corrected for radioactive decay on the day of each experiment. IPIN was used within one half-life and radiochemical purity was maintained as determined by ascending thin layer chromatography in 1:1 toluene:diethylamine. TLC plates were scanned using a Packard model 7220 radiochromatogram scanner.

Cell suspensions. In order to minimize nonspecific radioligand binding to culture plates and interstitial space, binding experiments were conducted in suspensions of intact cells. Culture medium was aspirated from tissue culture plates, and the cultures were washed twice with 4 ml of ice-cold assay buffer (which had composition identical to culture medium except that FCS and HCO_3^- were omitted and 4 mM HEPES, pH 7.4 was added). Sheets of cells were carefully scraped from the culture dishes with a rubber scraper and collected in a small volume of assay buffer. The intact cells were centrifuged at 500 × *g* at 4°, resuspended to a final concentration of approximately 0.3 mg of protein/ml and used immediately in the assay.

When membranes of cells were to be prepared, plates were washed with assay buffer; then the plates were vigorously scraped with a rubber scraper and cellular material was collected in a small volume of buffer. Cells were further disrupted by two freeze-thaw cycles followed by three washes with assay buffer or by treatment with a Polytron homogenizer (5 sec, 50% speed); control experiments showed identical receptor properties for either method. Final homogenization and resuspension were accomplished by 10 strokes of a tight-fitting Dounce homogenizer.

Cells used in radioligand-binding studies were grown in the presence of [^{75}Se]methionine to permit normalization for protein content of each sample using double label-counting techniques. Protein concentrations were estimated by the method of Lowry *et al.* (24) using bovine serum albumin as standard.

The viability of the intact cell preparation was estimated by trypan blue exclusion and by assay of LDH release into the supernatant phase (25, 26).

Receptor binding assay. β-Adrenergic receptor properties were assessed by adding 900 μl of intact cell suspension or cell membranes to a mixture containing the desired concentration of IPIN and, depending on the experimental design, β-adrenergic agonists or antagonists. To prevent oxidation, catecholamines were prepared and maintained in 1 mM ascorbate. Control experiments showed that ascorbate did not perturb IPIN binding. The binding reaction was conducted for varying periods of time at 37°. Binding was terminated by adding 3 ml of wash buffer (potassium phosphate, pH 7.4, 4°) to the assay tube followed by rapid filtering under controlled vacuum through Whatman GF/C filters. The filters were then washed twice with an additional 5 ml of buffer. Approximately 8 sec were required to terminate the binding

[1] The abbreviations used are: IPIN, iodopindolol; Gpp(NH)p, guanosine 5′-(β, γ-imido)triphosphate; LDH, lactic dehydrogenase; IHYP, iodohydroxybenzylpindolol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

reactions and wash the filters. The filters were dried under high vacuum and counted at 69% efficiency in a Searle gamma counter. Simultaneously determined ^{125}I counts (for IPIN binding) and ^{75}Se counts (to correct for intertube variation in cell density) were analyzed on a DEC PDP 11/23 computer.

Cyclic AMP measurements. To assess alterations in cellular cAMP content, intact cells on culture plates were exposed to graded concentrations of isoproterenol at 37° in the same medium that was used for binding experiments and contractility studies; phosphodiesterase inhibitors were not used. Cyclic AMP production was stopped at 3 min by addition of 10% trichloroacetic acid, preliminary experiments having shown peak cAMP content by this time. Cells and supernatant were harvested and cAMP was assayed by the method of Harper and Brooker (27).

Contractility experiments. To determine contractile state of cultured heart cells at baseline and in response to interventions, measurements of the amplitude of contraction of individual cells in spontaneously contracting monolayers at days 3 or 4 in culture were conducted (3) using a phase contrast microscope-video detector system described in detail by Marsh *et al.* (3). Cells in a specially designed perfusion chamber were continuously superfused with a test solution at 0.97 ml/min, while being exposed to a 95% air, 5% CO_2 atmosphere at 37 ± 0.2°. Perfusate containing various test solutions was controlled by a series of syringe pumps. The medium bathing cells in a monolayer could be changed with a half-time of about 30 sec as determined by indocyanine green washout measurements. The medium in which the contractility experiments were conducted was culture medium containing 0.6 mM calcium. Previous studies from this laboratory have demonstrated that this method of assessing the inotropic state of cells in a monolayer correlates very closely with other physiologic parameters known to relate to the inotropic state such as alterations in monovalent and divalent cation fluxes (28) and response to altered oxygen tension (5). The contractility measurements were made on only one cell per coverslip. Several coverslips were used from one plating; all experimental points included results from two or more platings.

Data analysis. Kinetic data were analyzed by the linear least squares method. Equilibrium binding data and agonist or antagonist competition data were analyzed using the iterative, nonlinear, least squares method of Munson and Rodbard (29) on an IBM 370 computer to solve the problem of the interaction of hormone with receptor using the law of mass action: $H_i + R_j > HR_{ij}$. This approach requires no data transformation as is necessary for conventional Scatchard analysis and permits improved estimation of nonspecific binding. When the model is applied to the case of separate affinity states, one assumes that these states, though interconvertible, can be nevertheless segregated. Considerable evidence supports these assumptions in some β-adrenergic receptor systems (10, 19, 20). Criteria used for goodness-of-fit were those of Kent *et al.* (20). Each experiment was analyzed individually and binding parameters for replicate experiments were averaged. Simultaneous analysis of all replicate experiments was also performed; binding parameters estimated in this fashion were in good agreement with the values obtained by averaging.

Materials. (−)-Pindolol was the generous gift of Dr. Joel Linden and of Sandoz Pharmaceuticals. ^{125}I was from New England Nuclear. [^{75}Se] Methionine was from Amersham. Gpp(NH)p was from Boehringer Mannheim. Tissue culture media were from sources previously described (3).

RESULTS

Intact cell preparation. At 3 to 4 days in culture, at least 70% of cells in the monolayer contracted spontaneously. To confirm that cells that had been suspended and prepared for radioligand-binding studies remained intact and viable, three sets of observations were made. Cells that had been harvested by gentle scraping, centrifugation, and resuspension in assay buffer were rewarmed to 37° and examined under a phase contrast microscope. Ten to 15% of the cells spontaneously contracted under these circumstances. Although macroscopically the cells appeared to be in uniform suspension, microscopically the cells remained in large sheets, and on average 85% of the cells thus prepared excluded trypan blue. Finally, cells that had been harvested and resuspended in assay buffer were centrifuged at 500 × *g* for 10 min and the LDH activity of the supernatant was assayed. An identical aliquot of cells was disrupted by 20 strokes of a tight fitting Dounce homogenizer and then centrifuged at 4000 × *g*. The supernatant was assayed for LDH as well. The supernatant of the intact cell preparation had an LDH activity of 10–15% (in three experiments) of the supernatant of the homogenate, in agreement with the estimate of membrane integrity by trypan blue exclusion.

To determine if the intact cell suspension permitted rapid washout of radioligand from the interstitial space (thus permitting low nonspecific binding), the intact cell suspension was incubated for 15 min with [^{51}Cr]EDTA as an extracellular space marker. Aliquots of the suspension were rapidly washed for varying periods of time and filtered as described in Materials and Methods. More than 99% of the ^{51}Cr counts washed out in less than 2 sec, indicating that the extracellular matrix that persisted in the sheets of suspended cells was not trapping the radioligand.

Antagonist binding. When intact cells were incubated for 15 min with increasing concentrations of the antagonist IPIN, binding was saturable (Fig. 1A). At concentrations of IPIN near the K_D, binding was 85% specific, with specific binding defined as that displaced by 1 μM (−)-propranolol (see below). When graphically presented as a Scatchard plot (Fig. 1B), it is apparent that the plot is linear and shows saturability with a dissociation constant of 22 pM. Preliminary binding experiments conducted with cells attached to culture dishes revealed unacceptably high nonspecific binding, due in part to ligand adherence to plastic.

Similarly, IPIN bound with a high degree of specificity (85% specific binding at the K_D) to membranes of cultured cells. The K_D for binding to cell membranes (39 pM) was statistically indistinguishable from that for whole cells or for homogenates plus 0.1 mM Gpp(NH)p (19 pM). The number of receptors per mg of protein was somewhat enriched in the membrane preparation (Table 1).

To confirm that binding of IPIN to the intact cells was stereospecific, the intact cells were incubated with IPIN plus varying concentrations of (−)-propranolol, (−)-alprenolol and (+)-propranolol. The levo isomer of propranolol was more potent than the dextro isomer in displacing IPIN from the intact cell's β-receptor. This stereospecificity persists at 1 μM but is no longer evident at 10 μM antagonist. Accordingly, the specific binding for these experiments was defined as that which is displaceable by 1 μM propranolol.

To determine if the receptor being identified had the expected potency series for a β-adrenergic receptor, intact cells were incubated with IPIN and varying concentrations of levo isomers of three agonists (Fig. 2). (−)-

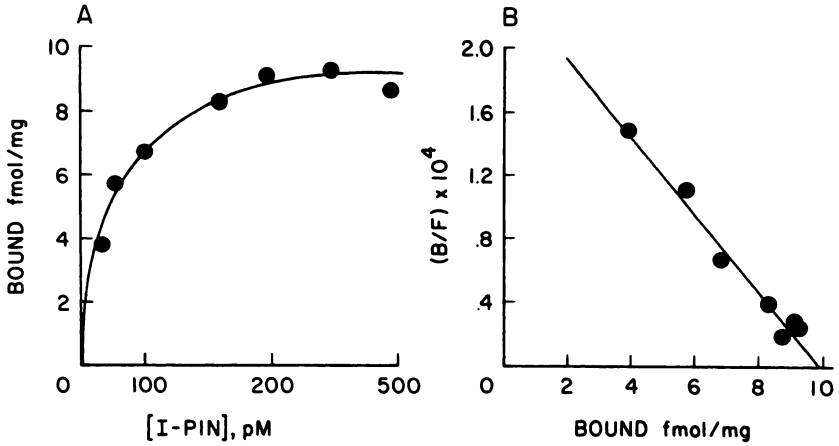

FIG. 1. *Equilibrium binding of IPIN to intact cells*

A, representative binding isotherm demonstrating specific, saturable binding. The binding was 80% specific at 50 pM. B, Scatchard plot of data presented in A. The K_D = 41 pM with B_{max} = 9.9 fmol/mg of protein for this typical experiment (r = 0.98).

TABLE 1

[^{125}I]Iodopindolol equilibrium binding

Results of 3–5 experiments, each determined in duplicate or triplicate (mean ± SD). K_D and B_{max} values were determined by computer analysis of binding data as described in the text.

	K_D	B_{max}	Receptors/cell[a]
	pM	*fmol/mg*	
Intact cells	22 ± 3	10.3 ± 2.1	2900
Membranes	39 ± 12	17.1 ± 6.3	

[a] Based on 2.1 × 10^6 cells/mg of protein.

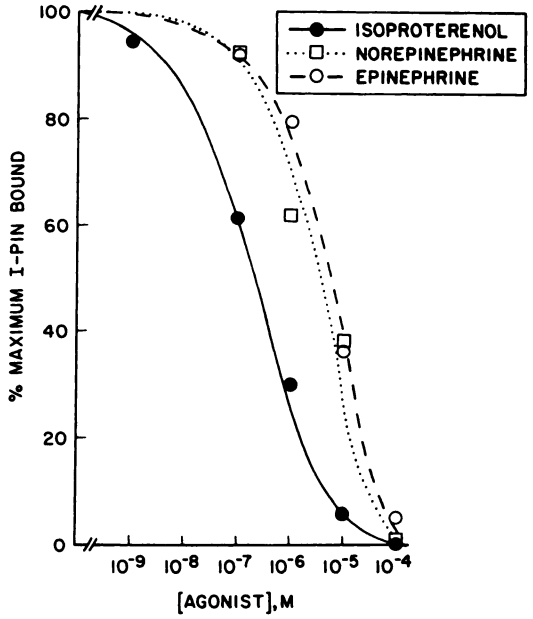

FIG. 2. *Competition for IPIN binding by agonists*

In this typical experiment, norepinephrine and epinephrine are equipotent in competing with 10 pM IPIN binding in intact cells. 0.1 mM isoproterenol was equipotent with 1 μM (−)-propranolol in competing for IPIN binding.

Isoproterenol was the most potent in competing for IPIN binding. Computer analysis of the (−)-norepinephrine and (−)-epinephrine competition curves yielded a K_D of 1.3 μM for epinephrine and 3.9 μM for norepinephrine. These dissociation constants were statistically indistinguishable. Thus, the receptor on the intact cells had the characteristics of a β_1-adrenergic receptor.

Binding kinetics. An independent method of estimating the affinity of IPIN for the receptor is to determine the kinetics of association and dissociation of IPIN from the β-receptor at 37°. Pseudo-first order kinetics can be assumed for the association reaction as less than 1% of the total ligand in the incubation mixture becomes bound. Intact cells were incubated for varying periods of time with 10 pM IPIN in the presence or absence of 1 μM propranolol. Binding was rapid, with equilibrium being approached by 3 min. When binding is plotted as a log function, k_{obs} can be estimated from the slope of the line, with $k_{obs} = k_1(L) + k_2$ where k_{obs} is the rate constant for the observed association reaction, k_1 is the pseudo-first order association rate constant, k_2 is the first order dissociation rate constant, and (L) is the concentration of the radioligand. To determine kinetics of dissociation of IPIN from the receptor, intact cells were incubated for 15 min with 10 pM IPIN in the presence or absence of 1 μM propranolol. After 15 min of preincubation, the reaction mixture was diluted 10-fold with assay buffer containing 10 μM propranolol, and the incubation was continued at 37° for varying periods of time. Aliquots were taken and rapidly filtered and washed. Dissociation of specific binding at 37° was rapid. An estimate of k_2 can be obtained from the slope of the log plot of the fraction bound as a function of time. With k_2 thus calculated, k_1 was calculated as $(k_{obs} - k_2)/L$ and K_D was calculated as k_2/k_1. The average k_{obs} = 0.697 min^{-1} (three experiments) and the average k_2 = 0.603 min^{-1}; $k_1 = 9.4 \times 10^9$ min^{-1} M^{-1}. Thus, the kinetically derived K_D for IPIN is 64 pM which is reasonably comparable to 22 pM derived from equilibrium binding isotherms.

Agonist binding. To characterize the interaction of a

β-adrenergic agonist with intact cultured heart cells, cells were incubated with 10 pM IPIN and varying concentrations of isoproterenol for 5 min. Isoproterenol competed with IPIN binding in a concentration-dependent fashion with 10 μM (−)-isoproterenol competing with as much radioligand as 1 μM (−)-propranolol (Fig. 3). Computer analysis of five such agonist-antagonist competition experiments in intact cells indicated that the interaction of the agonist with the β-receptor could be modeled best by a single site or receptor state ($p < 0.05$). Furthermore, the slope factor for the interaction of isoproterenol with the β-receptor in the intact cells was indistinguishable from 1, suggesting a single receptor population with no discernable cooperative effects (Table 2). Of note, the dissociation constant for isoproterenol in intact cells was 0.28 μM, a concentration almost 2 orders of magnitude higher than the EC_{50} for augmentation of contractility or for stimulation of cAMP accumulation in the same preparation (see below). Accordingly, we sought to determine if a higher affinity state of the β-receptor might be present in the absence of the GTP pool present in the intact cells. The interaction of isoproterenol with the β-receptor in membranes prepared from the cultured cells was determined under identical conditions. Competition of IPIN from membranes (Fig. 3) showed a more shallow curve than that of intact cells. The slope factor for the membranes was 0.76 ± 0.07, suggesting more than one receptor state. By computer analysis, a high affinity state of the β-adrenergic receptor for agonist was identified with a K_D of 3 nM. Binding data for membrane experiments were fit best by a two-receptor state model ($p < 0.01$); in no case was a one-site model statistically preferred.

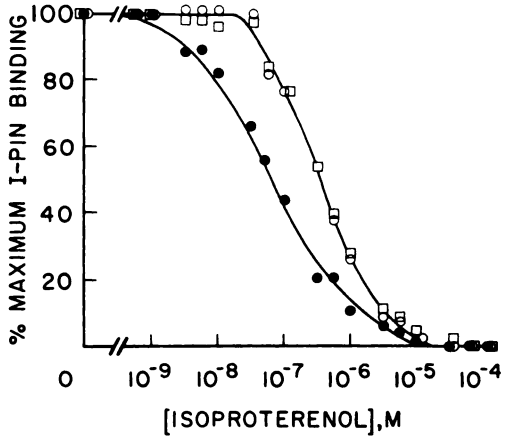

FIG. 3. *Competition for specific IPIN binding by (−)-isoproterenol under equilibrium conditions*

●, homogenate; ○, intact cells; □, homogenate plus Gpp(NH)p. Curves are typical of those obtained from three to seven experiments under each condition.

TABLE 2

Effect of guanine nucleotides on agonist and antagonist affinity

Dissociation constants[a]	Membranes	Intact cell	Homogenate (0.1 mM Gpp(NH)p
$K_{D\text{-IPIN}}$ (pM)	39 ± 12	22 ± 3	19 ± 7
$K_{H\text{-ISO}}$ (nM)	3 ± 2		
$K_{L\text{-ISO}}$ (μM)	0.31 ± 0.26	0.28 ± 0.09	0.52 ± 0.15
R_H/R_L[b]	55/45	−/100	−/100
N_H[c]	0.76 ± 0.07	1.14 ± 0.22	0.91 ± 0.13

[a] Results of simultaneous computer analysis of three to four 18-point agonist competition curves for each experimental condition. Dissociation constants are the reciprocals of the association constants produced by computer estimate. ISO, isoproterenol.

[b] Portion of receptors in low and high affinity state.

[c] Slope factor (mean ± SD).

To determine explicitly if agonist binding to the β-receptor in this system is modulated by guanine nucleotides, membranes of cells were incubated in the presence of 0.1 mM Gpp(NH)p, 10 pM IPIN, and graded concentrations of isoproterenol (Fig. 3 and Table 2). The addition of exogenous guanine nucleotide reverted agonist-binding properties to a state indistinguishable from that of the intact cell; only one low affinity receptor state could be identified ($p < 0.05$) with the slope factor approaching 1.0.

Recent studies in intact tumor cells have demonstrated that, if agonist binding is studied under very rapid, nonequilibrium conditions, it is possible to identify a higher affinity state for agonist than is apparent at equilibrium (11, 12, 18). Accordingly, intact heart cell suspensions were prewarmed to 37° and incubated with IPIN and increasing concentrations of isoproterenol for 15 sec, prior to rapid filtration and wash with 4° buffer in a fashion identical to that for equilibrium binding experiments. Shallow agonist displacement curves were observed ($n = 3$) (Fig. 4). For each of these 17-point agonist competition curves, binding was best characterized by two affinity states for agonist, with the mathematical model for two states being preferred at the level of confidence of $p < 0.01$ for each curve. The IC_{50} for high affinity binding was 1.1 ± 0.9 nM with a range of 0.24 ± 2.73 nM. Forty-seven ± 5% of receptors were in the high affinity state. Furthermore, when all curves are

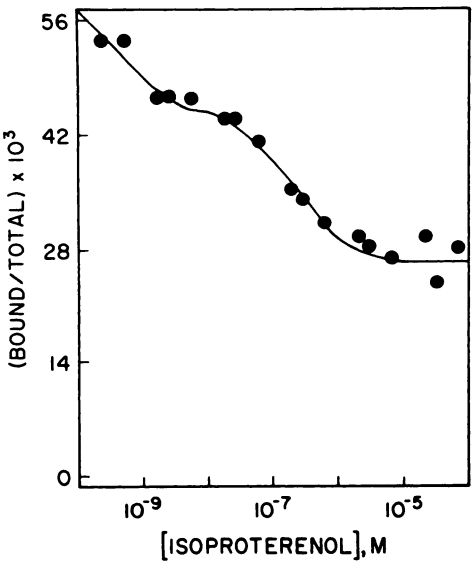

FIG. 4. *Competition for IPIN binding by (−)-isoproterenol at 15 sec at 37°*

Filled circles indicate observed binding (mean of duplicate determinations). The solid line represents the computer-generated best fit of the data to the mathematical model incorporating two binding states for agonist. Data are typical of three experiments.

analyzed simultaneously, two states are again preferred over the one-state model ($p = 0.001$). The $IC_{50} = 0.72$ nM for the high affinity state was determined in this fashion. To assess interexperiment variation in quality of curve fits, the parameters obtained from simultaneously analyzing all curves were compared to parameters obtained from analysis of each experiment individually. By both the F test and residuals test, no difference could be found between individual experiments and simultaneous analysis of all experiments ($p > 0.2$), indicating good reproducibility of the observations.

Cyclic AMP production. Isoproterenol-augmented cellular cAMP content in a concentration-dependent fashion. In the absence of a phosphodiesterase inhibitor, basal (unstimulated) cAMP was 2.5 ± 1.0 pmol/mg; 1 μM isoproterenol caused maximal increase in cAMP content of 18.2 ± 4.5 pmol/mg. When four 10-point concentration-effect curves were examined by log-logit analysis, the EC_{50} for stimulation of cAMP content by isoproterenol was found to be 4 nM.

Contractility measurements. To assess the relationship between β-adrenergic agonist concentration and physiologic response, increase in amplitude of contraction of spontaneously contracting myocytes in culture was determined as a function of isoproterenol concentration (Fig. 5). To minimize potential desensitization, no cell was exposed to more than three concentrations of isoproterenol and each exposure was for no more than 3 min (3). The maximal response observed at 1 μM isoproterenol concentration was typically two-thirds the increase in amplitude and velocity of cell motion that could be achieved with maximal stimulation with 3.6 mM Ca. The 1 μM isoproterenol response is plotted as 100% maximal response in Fig. 5. A log-logit plot of the concentration-effect relationship yielded an EC_{50} for isoproterenol-induced enhancement of contractility of 6 nM.

The fraction of receptor sites occupied at a given concentration may be calculated as $R_H/R_T = [H]/([H] + K_D)$, where R_H = number of occupied receptors/cell, $[H]$ = free hormone concentration, R_T = total number of receptors/cell, and K_D = dissociation constant for the hormone (30). This assumes that hormone binding has reached equilibrium, a condition likely met for R_H but probably not for R_L (12). Fig. 6 shows the full relation of calculated fractional occupancy of the high and low affinity receptor state of intact cells to contractile response and EC_{50} for cAMP accumulation. There is good correspondence between high affinity receptor occupancy, contractile response, and stimulation of cAMP content. There is statistically significant stimulation of contractility with 0.1 nM isoproterenol (8% of maximal response). If occupancy of R_H mediated this physiologic response, occupancy of 10% of high affinity receptors would produce 8% maximal physiologic response. Conversely, if the contractile response was mediated by occupancy of the low affinity state as defined in homogenates at equilibrium, 0.03% of receptors, or about 1 receptor/cell, would be occupied. To the extent that hormone is not at equilibrium with either state of the receptor, the K_D is overestimated, and estimated receptor occupancy would be greater at a given concentration.

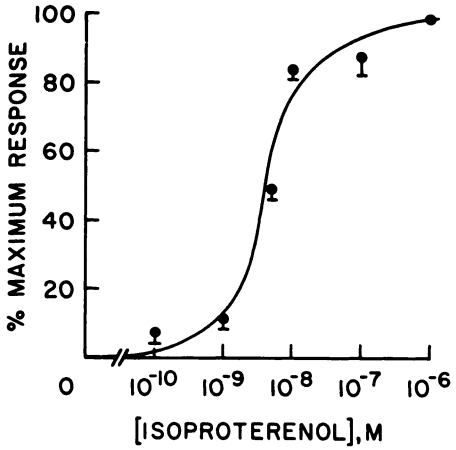

FIG. 5. *Contractile response of intact cells to isoproterenol*
Percentage of maximal increase in amplitude of spontaneous contraction is expressed as a function of isoproterenol concentration. Bars indicate standard error with n values of four to nine for each point.

DISCUSSION

Primary cultures of ventricular cells from embryonic chick heart have proven to be useful in physiologic investigations of cardiac control mechanisms (3, 4, 6, 28). We have now characterized the β-adrenergic receptor in the intact cultured heart cell as well as membranes of the cell and related the binding of an agonist to cAMP production and physiologic contractile response. During embryogenesis, there is considerable fluctuation in adrenergic neurotransmitter concentrations in avian heart (1). For chick embryos at 10 days *in ovo*, however, the number of β-adrenergic receptors is at a plateau (2). Agonist and antagonist binding to adrenergic receptors on intact cells cultured from chick hearts at this stage has not been previously characterized. These observations support the view that two affinity states of the receptor exist and that binding is modulated by guanine nucleotides.

The suspension of cultured cells used for characteri-

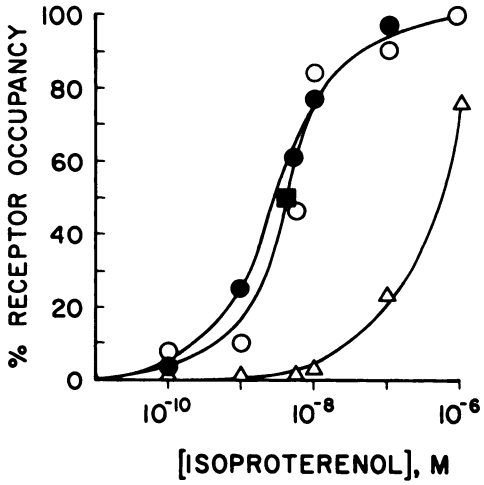

FIG. 6. *Relation of physiologic response to receptor occupancy*
Contractile response to isoproterenol (○), replotted from Fig. 4, is compared to calculated occupancy of the high affinity receptor state of intact cells (●) and low affinity receptor state (△). EC_{50} for increase in cAMP content is also shown (■).

zation of the β-adrenergic receptor consisted mainly of intact, viable cells and thus was directly comparable to the preparation on which cAMP production and contractility studies were performed. The experimental device of using cell suspensions obviated potential problems of extracellular space washout and permitted rapid separation of cells from unbound ligand, avoiding the need for prolonged washing steps required for cells attached to plates (31) during which alteration of specific hormone binding may occur. Cell viability was documented by the ability of harvested cells to continue to beat spontaneously, ensuring intact membranes and cells with minimally altered electrophysiologic properties. Furthermore, trypan blue exclusion observations and LDH assay of the supernatant of the intact cells gave concordant results indicating viability of at least 85% of the cells (26). Cell sheets were suspended without enzymatic treatment or exposure to low Ca concentrations to keep membrane properties unperturbed. This differs from other studies on cultured heart cells and may account for the disparate results (7, 9).

The electrolyte composition of the medium in which the receptor assays were conducted was identical to that for cAMP studies and that in which the contractility studies were performed; the receptor assay solution was buffered by HEPES rather than bicarbonate, but we have observed similar results of contractility experiments in either buffer (3, 28). Taken together, these methodologic considerations provide a sound rationale for comparison of biochemical and physiologic findings.

Previous investigators have examined antagonist binding to the β-receptor in cultured heart cell membranes (6, 7) but delineation of binding to intact cells using the ligand IHYP was not successful (7). Lau *et al.* (9) were able to identify β-adrenergic receptors in presumably intact cardiac myocytes, but under conditions (total absence of calcium) that were not physiologic and therefore did not permit a direct correlation of ligand-binding data with physiologic performance. These limitations have now been overcome; our binding experiments were conducted using intact cell suspensions at 37° in the presence of a physiologic salt solution. Recently Porzig *et al* (8) reported antagonist and agonist binding to intact myocardial cells in culture medium, but analysis did not permit resolution of multiple receptor states. Working with other cultured cell systems, other investigators have been successful in characterizing adrenergic receptors in intact cells (8, 11, 12, 18, 23), but have not reported results from a system in which binding experiments were conducted under physiologic conditions or where a physiologic response other than augmentation of cAMP content was measured (32, 33).

Intact animal and human studies have suggested that pindolol may possess partial agonist activity. It is very unlikely in this preparation, in the concentrations used, that partial agonist activity confounded analysis of binding. Indeed, Wolfe and Harden (34) found no agonist activity of pindolol in a muscle cell line using concentrations up to 10 μM. In the current study, guanine nucleotides (either exogenous or endogenous) did not lower pindolol affinity as they did for another agonist (isoproterenol). The small shift in IPIN affinity associated with guanine nucleotide was toward higher affinity as was reported in a myoblast cell line by Wolfe and Harden (34).

The receptor identified using IPIN has the expected properties of a β_1-adrenergic receptor: binding is saturable and stereospecific, with a relatively low amount of nonspecific binding. The potency series for displacement of agonists and antagonists is that expected of a β_1 receptor, although studies reported here do not exclude with complete certainty the presence of any β_2 receptor. Estimates of the dissociation constant for IPIN determined by equilibrium binding isotherms agree reasonably well with the dissociation constant derived kinetically. The achievement of a high degree of specific binding under physiologic conditions required the use of iodopindolol, which is less lipid soluble than dihydroalprenolol or IHYP. Indeed, despite extensive efforts, we were unable to demonstrate satisfactory specific binding of the commercially available IHYP to intact cultured heart cells. Taken together, these observations validate the study of the β receptor in viable, physiologically active cultured heart cells and cell membranes using IPIN.

In addition to characterizing antagonist binding to the β receptor of intact myocytes under physiologic conditions, this cultured cell system permitted study of the interaction of agonist with the β receptor and its relation to physiologic response. As expected on the basis of work in other experimental systems where formation of a GTP-dependent high affinity ternary complex is a crucial intermediate (10, 20), at equilibrium, agonist displacement curves utilizing intact cells (containing GTP) did not demonstrate the high affinity state of the β-adrenergic receptor that we demonstrated in homogenates of these cells. If formation of the high affinity state of the receptor in the intact cell were followed very rapidly by conversion of the receptor to the low affinity state in the presence of GTP, with concomitant activation of adenylate cyclase, then the high affinity binding state would be present in only catalytic quantities, would not accumulate, and thus would be difficult to detect in intact cells or in the presence of exogenous guanine nucleotides (12, 20).

We found that if agonist binding was measured very rapidly, a high affinity state of the receptor could be identified in intact cells. The IC_{50} (1.1 nM) determined in this fashion was comparable to K_H in washed membranes (3 nM). The validity of estimating the affinity for agonist under nonequilibrium conditions is dependent on conditions approximating the initial velocity of binding of the radioligand (12, 18). In that our binding conditions and ligands were identical to those where initial velocity conditions have been demonstrated to be met (12, 18), the IC_{50} of 1.1 nM should represent a reasonable estimate of the high affinity state of the receptor for agonist. Furthermore, when an identical intact cell assay is conducted at 4° for 1 hr, the high affinity state (IC_{50} = 0.26 nM) is also preserved (data not shown).

As predicted from the nonequilibrium binding model (18), the IC_{50} for low affinity binding to intact cells at

37° for 15 sec (1.1 ± 0.9 μM) is somewhat higher than that found in homogenates (0.31 μM) under equilibrium conditions.

The current studies identified a substantially higher affinity state of the β-receptor for agonist in cultured heart cells than has been reported from some laboratories (200–2000 nM) (7–9). However, utilizing a sarcolemmal preparation from rat hearts, Davies *et al.* (35) reported two affinity states for isoproterenol, with the higher affinity state showing a K_D of 12 nM and a lower affinity K_D of 350 nM, values similar to those reported here for cell homogenates.

Although a relatively rapid (5 min) binding assay was utilized, and the contractile concentration-effect curve was determined under conditions where contractile desensitization has been shown to be minimal (3), it is possible that agonist-induced desensitization of the receptor in the intact cell was occurring in the course of the assay (11, 12). While receptor desensitization in membranes has been reported to occur (36), it is likely not a major problem during the 5-min membrane assay (15).

The present study demonstrates the presence of two affinity states of the β-adrenergic receptor in cultured heart cells. It appears that occupancy of a high affinity state of the receptor by agonist stimulates cAMP production and mediates the augmentation of contractility. The concentration-effect relationships among high affinity hormone binding, cAMP production, and physiologic response of the intact cell are close (Fig. 6). The close associations among EC_{50} for stimulation of cAMP content, EC_{50} for contractility, and K_D for the high affinity state in intact cells or homogenates are consistent with the lack of a large pool of spare receptors in this system under the conditions studied, although the present study was not designed to resolve conclusively the issue of the efficiency of the coupling between β receptor, adenylate cyclase, and physiologic response for the heart.

Strickland and Loeb (37) have considered possible theoretical relations between hormone binding and physiological response in systems where there are mediators (such as cAMP) interposed between effector and response. In systems in which the concentration of such mediators achieved at maximum receptor occupancy is much less than the equilibrium constant for intermediate reactions such as Ca channel phosphorylation, then the K_D for receptor occupancy and EC_{50} for physiologic effect will be nearly identical. Conversely, if much less than maximal receptor occupancy produces concentrations of mediators in excess of the dissociation constant for intermediate reactions, then the EC_{50} for physiologic response will be less than the K_D for hormone binding. The intermediate effector systems for β-adrenergic agonist effects on cardiac cells are complex, so exact agreement between K_D and EC_{50} is not *a priori* to be expected. The data reported here are, however, entirely consistent with a model in which the concentrations of intermediate effectors achieved are less than the dissociation constants for their reactions. Isoproterenol produced a half-maximal increase in cAMP content at 4 nM; this concentration is in reasonable agreement with the K_D of isoproterenol for the high affinity state and the EC_{50} (6 nM) for contractility. Further study of the kinetics and mechanisms of agonist-induced receptor regulation in intact cultured heart cells is necessary to provide additional support for the thesis that the high affinity state of the receptors found in membrane is the physiologically active state in intact cells.

Detailed chemical characterization of the β-adrenergic receptor-guanine nucleotide regulatory protein-adenylate cyclase complex is proceeding in several laboratories, and efforts at purification of components of this enzyme complex have met with considerable success (38). New insights into receptor internalization are also rapidly emerging (21). The observations reported here in intact, beating myocardial cells and myocardial cell membranes serve to complement these biochemical observations and place them in physiologic perspective. In an effort to relate receptor occupancy to function, we have demonstrated two affinity states of the β-adrenergic receptor in membranes of the cultured cells and using nonequilibrium binding methods, in intact cells as well. Hormone binding to the high affinity state of the receptor correlates closely with both the stimulation of cAMP production in intact cells and the physiologic contractile response in the same preparation. The identification and characterization of this potentially physiologically important receptor state will provide a framework within which to examine physiologic and pathophysiologic β-adrenergic receptor regulation in the heart.

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Send reprint requests to: James D. Marsh, M.D., Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115.