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Analysis of the Role of Receptor Number in Defining the Intrinsic Activity and Potency of Partial Agonists in Neuroblastoma × Glioma Hybrid NG108–15 Cells Transfected to Express Differing Levels of the Human β_2-Adrenoceptor

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SUMMARY

Many agonist ligands are known experimentally to display a range of efficacies and potencies in different tissues and preparations. To analyze the role of the levels of receptor expression and availability in the intrinsic activities and potencies of agonists, the function of a number of β-adrenoceptor ligands was examined in clones of neuroblastoma × glioma hybrid NG108–15 cells transfected to express differing levels of the human β_2-adrenoceptor, as well as after treatment of these cell lines with the irreversible β-adrenoceptor antagonist bromoacetyl alprenolol menthane (BAAM). Clone βN22 expressed approximately 10-fold higher levels of the receptor than did clone βN17. In measurements of agonist stimulation of adenylyl cyclase activity in membranes of these cells or agonist stimulation of the formation of the complex of $G_{s\alpha}$ and adenylyl cyclase, which acts as the high affinity binding site for [^{3}H]forskolin in whole cells, a series of β-adrenoceptor agonists, including dichloroisoprenaline, ephedrine, dobutamine, and salbutamol, displayed higher intrinsic activity and showed concentration-response curves that were substantially to the left (lower EC_{50} values) in clone βN22, compared with clone βN17. Treatment of clone βN22 cells with varying concentrations of BAAM reduced the intrinsic activity of these ligands and shifted the concentration-response curves for these agents to the right. In clone βN22 cells and membranes, reduction in the observed intrinsic activity for ephedrine required elimination of a smaller fraction of the β_2-adrenoceptor reserve than for salbutamol and reduction in the effect of the full agonists isoprenaline and epinephrine was noted only with high fractional elimination of the receptor pool. The effect of isoprenaline was substantially reduced, however, by BAAM treatment of clone βN17 cells, where the β_2-adrenoceptor number approached extremely low levels. Analysis of the data using the formalisms of Whaley *et al.* [*Mol. Pharmacol.* **45**:481–489 (1994)] showed that prediction of alterations in agonist potency with receptor number for full agonists can be adequately extended to partial agonists.

Partial agonists display lower intrinsic activity, compared with full agonists, as assessed by their inability to produce the maximal response of a system even at maximal receptor occupancy. The terms efficacy (1) and intrinsic activity thus relate to the maximal ability of a drug-receptor complex to evoke a response in a cell or tissue. However, receptor theory anticipates that the observed intrinsic activity of ligands also depends on the level of receptor expression and receptor reserve and potentially on the level at which the transmitted signal is measured (2, 3). Before the availability of cDNA species encoding G protein-linked receptors, measurements of differences in agonist efficacy and intrinsic activity could be gained only by comparing responses to an agonist in different tissues or by using irreversible antagonists to block varying proportions of the receptor population. Genetic manipulation of the β-adrenoceptor level in cells has been achieved (see Refs. 4–6 as examples); however, the conclusions regarding the influence of receptor levels on the intrinsic activity and the efficacy of adrenergic agonists are not consistent. Furthermore, both of these approaches have their inherent experimental limitations (see Ref. 2 for a review of some of these concerns).

As a means to examine the importance of receptor levels in defining the intrinsic activity and potency of agonist drugs without alteration of the amounts of the other components of the signal transduction cascade, herein we use a mixed experimental strategy, utilizing two clones of neuroblastoma × glioma hybrid NG108–15 cells that we have transfected to express different levels of the human β_2-adrenoceptor (7, 8).

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ABBREVIATIONS: BAAM, bromoacetyl alprenolol menthane; DHA, dihydroalprenolol; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Of these clones, βN22 cells express approximately 10-fold higher levels of this receptor than do cells of clone βN17, but the clones show no differences in immunologically detectable levels of either the stimulatory G protein $G_{s\alpha}$ (9) or adenylyl cyclase (10). We also chemically alter the effective β_2-adrenoceptor concentrations in these cells by treatment with the irreversible β-adrenoceptor antagonist BAAM.

We demonstrate that partial agonists show higher observed intrinsic activity in clone βN22, compared with clone βN17, as assessed by measuring either adenylyl cyclase activity in membranes of these cells or the ability of the ligands to stimulate the formation of a complex of $G_{s\alpha}$ and adenylyl cyclase in whole cells. Furthermore, after elimination of smaller fractions of the receptor population by treatment of the cells with the irreversible antagonist, the agonist properties of drugs with lower intrinsic activity are reduced more than are those of other drugs with greater intrinsic activity. As anticipated from our previous calculation of the low levels of the adenylyl cyclase catalytic moiety in these cells (9), clone βN22 displays a considerably greater receptor reserve, compared with clone βN17, which is observed as a leftward shift in the concentration-response curve (lower EC_{50} values) in either of these assays. This effect is reversed by eliminating proportions of the receptor population. Such data are then analyzed by the formalisms of Whaley *et al.* (4), to extend their examination of the role of β-adrenoceptor number and the regulation of adenylyl cyclase activity to include data for partial agonists.

Experimental Procedures

Materials

All reagents for tissue culture were purchased from Gibco/BRL. [^{3}H]Forskolin (36 Ci/mmol), [^{3}H]DHA (56 Ci/mmol), [α-^{32}P]ATP, and [^{3}H]cAMP were obtained from Amersham International. BAAM and the other β-adrenoceptor compounds were from Research Biochemicals International (Natick, MA). All other chemicals were bought from Sigma or BDH and were of the highest purity available.

Methods

Generation and isolation of clones of NG108–15 cells expressing the human β_2-adrenoceptor. Plasmid pJM16 (11), which harbors a copy of the neomycin resistance gene, was cut with the restriction enzymes *Bam*HI and *Xho*I to allow a cDNA encoding the human β_2-adrenoceptor to be ligated downstream of the β-actin promoter of this plasmid (7, 8). Human β_2-adrenoceptor cDNA (treated with Geneclean; Gibco/BRL), with 5′ *Bam*HI and 3′ *Xho*I sites, was ligated into the digested pJM16. Competent *Escherichia coli* were transformed with DNA from the ligation reactions and coated onto ampicillin (25 μg/ml)-containing plates, and ampicillin-resistant colonies were picked. Ten micrograms of this DNA were transfected into NG108–15 cells using Lipofectin reagent (Gibco/BRL), according to the manufacturer's instructions. Clones that were resistant to Geneticin sulfate (800 μg/ml) were selected and expanded. Expression of the β_2-adrenoceptor in membranes from these clones was assessed by the specific binding of the β-adrenoceptor antagonist [^{3}H]DHA (see below). The generation of clones βN17 and βN22 and the characterization of receptor expression by these clones have been described previously in detail (7, 8).

Cell growth. Transfected neuroblastoma × glioma hybrid NG108–15 cells were grown in tissue culture as described previously (7, 8). Before confluency, they were either split 1:10 into fresh tissue culture flasks or harvested. In a number of instances cells were treated with varying concentrations of BAAM for 4 hr before cell harvest.

Membrane preparation. Membrane fractions were prepared from cell pastes that had been stored at −80° after harvest, essentially as described (12). Frozen cell pellets were suspended in 5 ml of 10 mM Tris·HCl, 0.1 mM EDTA, pH 7.5 (buffer A), and the cells were ruptured with 25 strokes of a hand-held Teflon/glass homogenizer. The resulting homogenate was centrifuged at 500 × *g* for 10 min, in a Beckman L5–50B centrifuge with a Ti 50 rotor, to remove unbroken cells and nuclei. The supernatant fraction from this treatment was then centrifuged at 48,000 × *g* for 10 min, and the pellet from this treatment was washed and resuspended in 10 ml of buffer A. After a second centrifugation at 48,000 × *g* for 10 min, the membrane pellet was resuspended in buffer A (to a final protein concentration of 1–3 mg/ml) and stored at −80° until required.

Adenylyl cyclase assays. These assays were performed as described by Milligan *et al.* (13). Each assay contained 100 mM Tris·HCl, pH 7.5, 20 mM creatine phosphate, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM cAMP, 1 μM GTP, 10 units of creatine phosphokinase, and 0.2 mM ATP including 1 μCi of [α-^{32}P]ATP. Separation of radiolabeled cAMP and ATP was achieved using the double-column method described by Johnson and Salomon (14).

Cell preparation for [^{3}H]forskolin binding experiments. The specific high affinity binding of [^{3}H]forskolin to the $G_{s\alpha}$-adenylyl cyclase complex in whole cells was determined essentially as described by Kim *et al.* (10) and Alousi *et al.* (15). Cells were removed from tissue culture flasks, counted using a hemocytometer, resuspended in HEPES-buffered Dulbecco's modified Eagle's medium (buffer B), and added to tubes containing [^{3}H]forskolin (approximately 10 nM) in either the absence or the presence of 10 μM nonradiolabeled forskolin to define nonspecific binding. The tubes also contained varying concentrations of the individual β-adrenoceptor ligands. Tubes were then incubated at 4° for 60 min, and the mixtures were filtered through Whatman GF/B filters, followed by liquid scintillation counting. In experiments that included BAAM treatment, cells were treated with the appropriate concentration of this agent for 4 hr and after cell harvest the cells were washed extensively with buffer B by a series of centrifugation and resuspension steps.

In a number of preliminary experiments, [^{3}H]forskolin binding was measured in whole cells in the presence of a receptor-saturating concentration of isoprenaline (10 μM) and displacement of this binding was achieved with differing concentrations of forskolin. Such displacement data were analyzed as described (10, 13), to allow estimation of the K_d for [^{3}H]forskolin binding to the $G_{s\alpha}$-adenylyl cyclase complex in whole cells.

Binding experiments with [^{3}H]DHA. In experiments designed to assess the maximal binding capacity of membranes for [^{3}H]DHA, concentrations of this ligand were varied between 0.1 and 5 nM, in the absence and presence of 10 μM propranolol to define maximal and nonspecific binding, respectively. Assays were performed at 30° for 30 min in 20 mM Tris·HCl, pH 7.5, 50 mM sucrose, 20 mM $MgCl_2$ (buffer C). Specific binding, defined as described above, represented >90% of the total binding of [^{3}H]DHA. All binding experiments were terminated by rapid filtration through Whatman GF/C filters, followed by three washes (5 ml) with ice-cold buffer C. In experiments designed to assess the effectiveness of a range of concentrations of BAAM in producing irreversible blockade of the β_2-adrenoceptor population, membranes prepared (as described above) from BAAM-treated cells were incubated (as described above) with a single concentration (2 nM) of [^{3}H]DHA, in the absence and presence of 10 μM propranolol to define maximal and nonspecific binding, respectively. The K_i values for the adrenergic agonists used here were determined using [^{3}H]DHA binding competition assays, as described by Cheng and Prusoff (16).

Data analysis. All binding data were analyzed using the Kaleidograph curve-fitting program (version 2.1) on an Apple Macintosh computer.

Results

Adenylyl cyclase assays were performed with membranes of both βN22 cells (which expressed the β_2-adrenoceptor at approximately 3000–4000 fmol/mg of membrane protein in individual passages of the cells) (7, 8) and βN17 cells (which expressed this receptor at approximately 10-fold lower levels) (7, 8), using a range of β-adrenoceptor ligands. Basal adenylyl cyclase activity varied approximately 2-fold in membrane preparations from individual cell passages (see figure legends for absolute adenylyl cyclase activities in individual experiments), an observation not unusual for cultured cell lines, but in the absence of receptor ligands the activity was routinely higher in membranes from βN22 cells than in those from βN17 cells (βN17 had 57.3 ± 9.4% of basal βN22 adenylyl cyclase activity, mean ± standard deviation, six experiments), as we reported previously (8). Concentration-effect curves for isoprenaline, salbutamol, dobutamine, and ephedrine with membranes from βN22 cells demonstrated different potencies of these ligands but indicated that the maximal effectiveness of stimulation of adenylyl cyclase that could be achieved by each of these agents was produced with concentrations at or below 10 μM (Fig. 1; Table 1). It was observed that isoprenaline (10 μM) stimulated adenylyl cyclase activity to similar maximal levels in membranes from cells of clones βN22 and βN17 (isoprenaline-stimulated adenylyl cyclase activity in βN17 cells was 82.0 ± 8.2% of that seen in βN22 cells, mean ± standard deviation, six experiments) (see also Ref. 7). Using the values obtained for isoprenaline (10 μM) as a reference (100%), the other β-adrenoceptor ligands were observed to display a range of intrinsic activities in these membranes (Fig. 2A). Clearly, however, the efficacies of chloroisoprenaline (βN22, 38.6 ± 5.6%; βN17, 8.9 ± 0.8%), ephedrine (βN22, 66.2 ± 2.9%; βN17, 20.0 ± 0.6%), dobutamine (βN22, 83.2 ± 3.7%; βN17, 42.0 ± 1.9%), and salbutamol (βN22, 101.9 ± 2.4%; βN17, 71.8 ± 1.9%) (mean ± standard error, three to 10 experiments for individual drugs) were significantly greater in membranes from clone βN22 than in those from clone βN17 (Fig. 2A; Table 1). Neither alprenolol or pindolol displayed an intrinsic activity of >10% in membranes of βN22 cells, and neither had detectable agonist activity in membranes of βN17 cells. An equivalent pattern, in which isoprenaline produced similar maximal stimulations in whole βN22 and βN17 cells, was observed when the ability of the ligands to induce specific high affinity binding of [^{3}H]forskolin to the complex of $G_{s\alpha}$ and adenylyl cyclase was assayed (Fig. 2B). When the same group of β-adrenoceptor ligands as used in the adenylyl cyclase assay was examined in this assay, a range of intrinsic activities was again observed (Fig. 2B), and once again the measured intrinsic activity of these agonists was routinely higher in cells of clone βN22 than in those of clone βN17 (Fig. 2B).

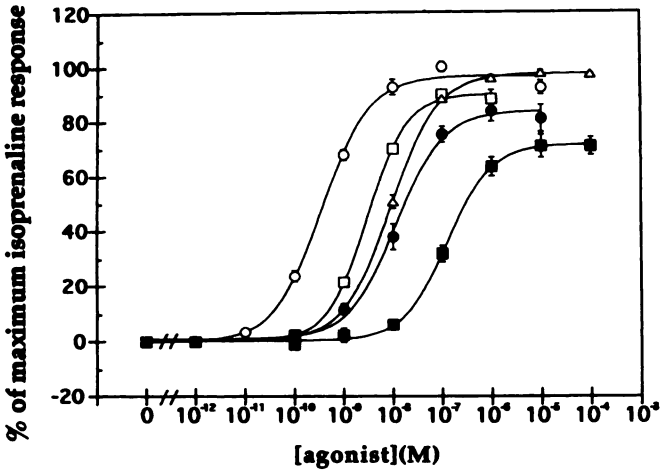

Fig. 1. Concentration-effect curves for stimulation of adenylyl cyclase in membranes of clone βN22 cells by β-adrenoceptor-active ligands. Membranes were prepared from clone βN22 cells and adenylyl cyclase activity was measured in response to differing concentrations of isoprenaline (○), epinephrine (□), salbutamol (△), dobutamine (●), or ephedrine (■). Results are presented as percentage of the maximal adenylyl cyclase activity response obtained with isoprenaline. In the experiments displayed, basal adenylyl cyclase activity in βN22 preparations was 53.7 ± 8.3 pmol/min/mg of membrane protein. Maximal isoprenaline-stimulated adenylyl cyclase activity was 214.2 ± 8.5 pmol/min/mg of membrane protein (mean ± standard deviation, five experiments).

Concentration-response curves for epinephrine indicated similar potencies in βN22 cells in the adenylyl cyclase and [^{3}H]forskolin binding assays (30.3 ± 3.1 nM and 7.5 ± 0.9 nM, respectively, mean ± standard deviation of triplicate determinations) (we previously noted that the [^{3}H]forskolin binding assay also resulted in a somewhat lower EC_{50} value for isoprenaline in cells of clones βN22 and βN17, compared with the adenylyl cyclase assay) (8, 10). Concentration-response curves for adenylyl cyclase stimulation in βN22 and βN17 cell membranes by isoprenaline, salbutamol, and ephedrine (Table 1) demonstrated that each drug had greater potency (as well as higher measured intrinsic activity in the cases of ephedrine and salbutamol) in membranes of βN22 cells than in those of βN17 cells. Again, a profile similar to that obtained in the adenylyl cyclase assay was generated by measurement of agonist-driven, high affinity [^{3}H]forskolin binding in whole cells. Concentration-response data for salbutamol-stimulated [^{3}H]forskolin binding demonstrated increased EC_{50} values and reduced intrinsic activity in βN17 cells, compared with βN22 cells (EC_{50} values of 660 ± 458 nM and 75 ± 7 nM and intrinsic activities of 77.0 ± 5.2% and 99.7 ± 2.9%, respectively, mean ± standard deviation of triplicate determinations).

If the difference in measured intrinsic activities of the β-adrenoceptor partial agonists between clones βN22 and βN17 was truly a reflection of differences in receptor levels between these two clones, rather than a trivial difference based on specific features of these particular clonal isolates, we predicted that functional ablation of varying proportions of the β_2-adrenoceptor population in cells of clone βN22 would decrease the potency and also the measured intrinsic activity of β-adrenoceptor partial agonists. βN22 and βN17 cells were treated with varying concentrations of the irreversible β-adrenoceptor antagonist BAAM. Membranes prepared from these cells were then assessed for the number of remaining specific [^{3}H]DHA binding sites (Fig. 3A). Half-maximal reduction in the number of available β_2-adrenoceptors was achieved by treatment with approximately 25 nM BAAM, and treatment with 1 μM BAAM reduced the number of β_2-adrenoceptor binding sites detected with [^{3}H]DHA by approximately 80%. Saturation binding studies using [^{3}H]DHA demonstrated that the effect of BAAM (100 nM) treatment was to reduce the available β_2-adrenoceptor pop-

TABLE 1

Summary of the potencies and efficacies of adrenergic agonists in βN17 and βN22 (with and without BAAM treatment) cell membranes

Full concentration-response curves were obtained with the adenylyl cyclase activity assay, to assess the potency (EC_{50}) and efficacy (intrinsic activity) of full and partial agonists under conditions of varying receptor number. BAAM (1 μM, 10 μM for isoprenaline experiments) pretreatment of βN22 cells and data analysis were as described in Experimental Procedures. Data represent the means ± standard deviations of between two and 10 separate experiments, each averaging triplicate determinations.

Agonist	βN22		βN17		βN22/BAAM	
	EC_{50}	Intrinsic activity	EC_{50}	Intrinsic activity	EC_{50}	Intrinsic activity
	nM	%	nM	%	nM	%
Isoprenaline	3.6 ± 0.6	100	100 ± 12	82.0 ± 8.2	310 ± 44	76.5 ± 1.8
Epinephrine	30.3 ± 3.1	90.1 ± 1.6	136 ± 75	83.4 ± 8.1	405 ± 61	95.0 ± 2.5
Salbutamol	109 ± 18	101.9 ± 2.4	449 ± 114	71.8 ± 1.9	882 ± 29	77.3 ± 0.4
Dobutamine	111 ± 27	83.2 ± 3.7	486 ± 121	42.0 ± 1.9	ND[a]	ND
Ephedrine	1,330 ± 300	66.2 ± 2.9	7,700 ± 667	20.0 ± 0.6	10,283 ± 1,667	17.5 ± 1.2

[a] ND, not determined.

ulation (B_{max}) in membranes prepared from the treated cells, without altering the measured affinity of the remaining receptors for [^{3}H]DHA (the K_d in control membranes was 0.84 ± 0.1 nM and that in membranes of BAAM-pretreated cells was 0.90 ± 0.23 nM) (Fig. 3B). Such data indicated that BAAM had been effectively removed during the production and washing of the membrane preparations and thus was not present to compete and potentially interfere in subsequent functional assays. Adenylyl cyclase assays performed with membranes derived from control and BAAM (10 μM)-treated βN22 cells demonstrated that, whereas there was a significant decrease in the potency of isoprenaline in the BAAM-treated cells, the intrinsic activity of isoprenaline was reduced only to approximately 80% of that observed in membranes from the untreated cells (Fig. 4A). Indeed, 1 μM BAAM treatment of βN22 cells resulted in a 14-fold shift in the epinephrine concentration-effect curve, with minimal diminution in the maximal epinephrine-stimulated adenylyl cyclase activity (Fig. 4A), demonstrating a receptor reserve. However, for salbutamol and ephedrine reductions in both potency and measured intrinsic activity were observed after BAAM (1 μM) treatment (Fig. 4B). Again, similar results were observed in whole cells when agonist stimulation of high affinity [^{3}H]forskolin binding was used as the functional assay (Fig. 4C). To examine this point in more detail, the intrinsic activities of isoprenaline, salbutamol, and ephedrine were measured with either adenylyl cyclase assays (Fig. 5A) or agonist-driven, high affinity [^{3}H]forskolin binding (Fig. 5B), using βN22 cells and membranes after treatment with a range of concentrations of BAAM for 4 hr. At the highest concentration of BAAM examined (10 μM), the measured intrinsic activity of isoprenaline was reduced, as noted above, by only approximately 15–20% in either assay but the measured intrinsic activities of the partial agonists ephedrine (10% of maximal isoprenaline response) and salbutamol (approximately 25% of maximal isoprenaline response) were reduced substantially, compared with those observed in untreated cells (Fig. 5). The intrinsic activity of ephedrine, which was demonstrated to be lower than that of salbutamol in untreated βN22 cells (Fig. 2), was clearly compromised after treatment with lower concentrations of BAAM than was that of salbutamol (Fig. 5). Furthermore, the data indicated that isoprenaline would require elimination of a greater amount of the receptor population than could be achieved in these experiments to significantly compromise its intrinsic activity (Fig. 5).

To examine to what extent the β_2-adrenoceptor population would have to be reduced to restrict the intrinsic activity of isoprenaline to 50% of that observed in untreated cells, cells of clone βN17 were treated with varying concentrations of BAAM. The ability of a concentration of isoprenaline sufficient to fully occupy the available receptors to stimulate adenylyl cyclase or to promote the specific high affinity binding of [^{3}H]forskolin was then assessed (Fig. 6). A reduction in the intrinsic activity of isoprenaline to 50% of that in untreated βN17 cells was achieved after treatment with approximately 2 μM BAAM. Because this concentration of BAAM was demonstrated to eliminate binding of [^{3}H]DHA to 90% of the expressed β_2-adrenoceptors on these cells (Fig. 3), this indicates that maximal occupancy of a β_2-adrenoceptor population of approximately 50 fmol/mg of membrane protein (corresponding to approximately 3000 receptors/βN17 cell) with this agonist would be sufficient to generate a half-maximal adenylyl cyclase response in these cells. To extend such analysis, we assessed the levels of receptor required to be occupied by each of isoprenaline, salbutamol, and ephedrine to obtain different degrees of intrinsic activity for these ligands (Fig. 7). Ephedrine, as noted above, failed to act as a full agonist even in membranes and cells of clone βN22, but 50% of the maximal possible receptor-mediated activation of adenylyl cyclase was achieved by occupancy by maximally effective concentrations of ephedrine of approximately 1800 fmol/mg of membrane protein of the β_2-adrenoceptor, whereas salbutamol required occupancy of approximately 500 fmol/mg of membrane protein of the receptor (Fig. 7). Such data demonstrate why isoprenaline and salbutamol act as full agonists in cells and membranes of clone βN22, whereas isoprenaline but not salbutamol is a full agonist in clone βN17. They further explain why ephedrine is not a full agonist in either system. It must be anticipated, however, that it would become so in clonal cell lines of this genetic background that express even higher levels of this receptor (5 pmol/mg of membrane protein and above).

To analyze the full and partial agonist dose-response relationships we observed here against the theoretical framework proposed by Whaley *et al.* (4) for alterations in agonist EC_{50} values with changes in receptor number, we determined the K_i values for the adrenergic agonists examined herein, using [^{3}H]DHA binding competition assays, the Cheng-Prusoff formalism (16), and a K_d value for [^{3}H]DHA determined to be 1.6 nM (data not shown). K_i values for isoprenaline, epinephrine, dobutamine, salbutamol, and

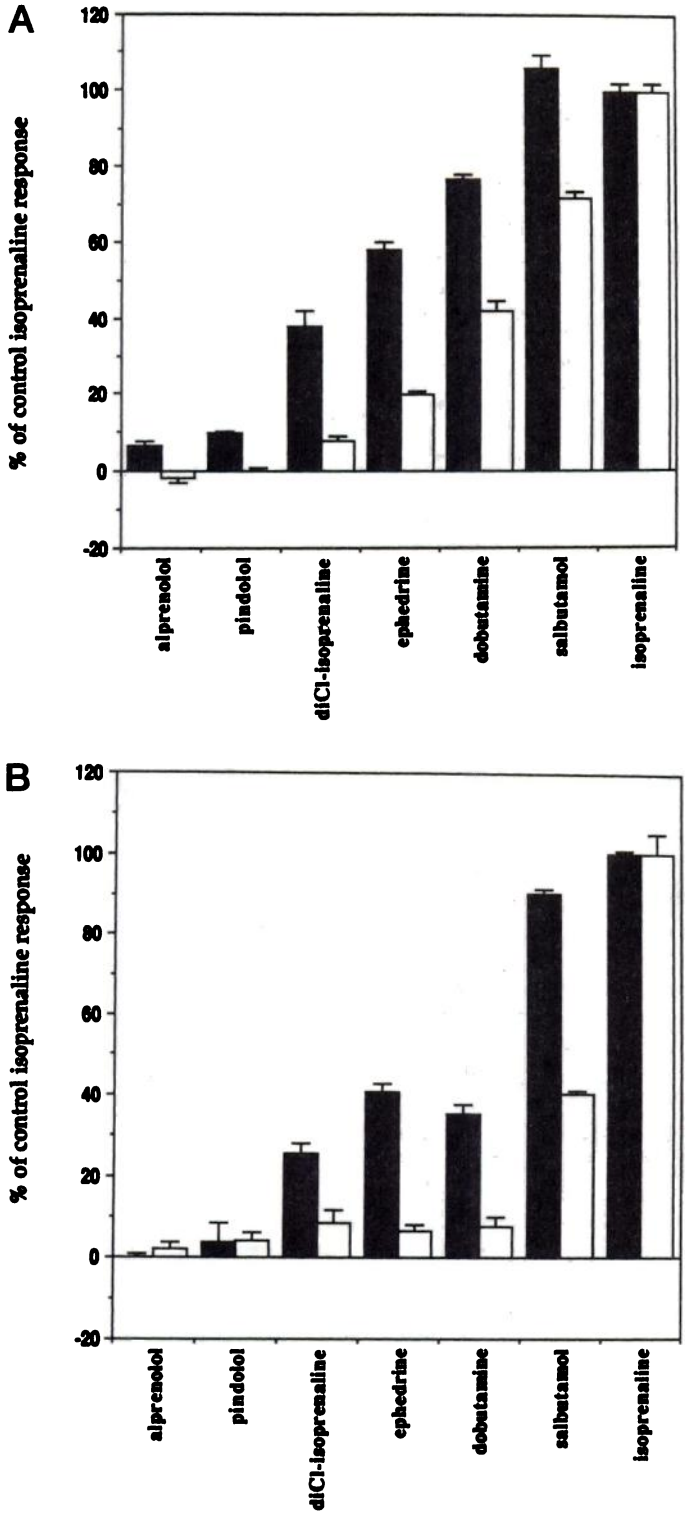

Fig. 2. Differences in intrinsic activities of β-adrenoceptor-active ligands when tested with cells expressing differing levels of the β_2-adrenoceptor. A, Adenylyl cyclase assays. B, High affinity [^{3}H]forskolin binding assays. Maximally effective concentrations of alprenolol, pindolol, dichloroisoprenaline, ephedrine, dobutamine, salbutamol, and isoprenaline (10 μM) were examined for their ability to stimulate adenylyl cyclase activity in membranes (A) or high affinity [^{3}H]forskolin binding in whole cells (3×10^5 cells/assay) (B) of either clone βN22 (■) or clone βN17 (□). Results are presented as percentage of the effect obtained with isoprenaline and represent the mean ± standard error of quadruplicate assays from a single experiment. Additional experiments produced similar results (see Results for details). In the experiments displayed, basal adenylyl cyclase activities in βN22 and βN17 preparations were 63.0 ± 8.3 and 43.0 ± 6.1 pmol/min/mg of membrane protein, respectively. Isoprenaline (10 μM)-stimulated adenylylcyclase activities in βN22 and βN17 preparations were 210.0 ± 12.8 and 155.1 ± 5.1 pmol/min/mg of membrane protein, respectively (mean ± standard deviation, two experiments). The nonspecific binding of [^{3}H]forskolin, as determined with excess nonradiolabeled forskolin, was routinely between 70 and 120 dpm/10^5 cells. Specific [^{3}H]forskolin binding was 90 dpm/10^5 cells in clone βN22 cells in the absence of any agonist and 580 dpm/10^5 cells in the presence of 10 μM isoprenaline; basal specific [^{3}H]forskolin binding accounted for 65 dpm/10^5 cells in clone βN17 cells and 540 dpm/10^5 cells in the presence of 10 μM isoprenaline.

ephedrine were calculated to be 0.68, 0.86, 2.2, 5.1, and 192 μM, respectively. Whaley *et al.* (4) proposed relationships between the potency (EC_{50}) or intrinsic activity (V_{max}) of a full agonist and the level of receptor expression. In our analysis, we have used their combined theoretical equation, $EC_{50} = (K_d \cdot k_{-1} \cdot V_{max})/(V_{100} \cdot k_1 \cdot r)$, where V_{max} is the maximal receptor-induced response of any agonist in the system, V_{100} is the maximal experimental response seen for a full agonist [in our system, the concurrently measured response of a maximal concentration (10^{-5} M) of isoprenaline], r is the number of receptors, and k_{-1} and k_1 are variables that relate to the efficiency of G protein-mediated signal transduction in a particular system. In the present analysis, we set k_{-1} and k_1 to the values 1 and 0.012, respectively, because these values provided the best fit to the experimental data. As can be seen in Fig. 8, the actual potencies of the full range of agonists and partial agonists under conditions of higher (βN22 cell membranes) and lower (βN17 cell membranes and BAAM-treated βN22 cell membranes) receptor levels were in good agreement with the predicted potencies calculated according to the method of Whaley *et al.* (4). Thus, not only do these theoretical equations hold true for full agonists across a range of cellular receptor levels, but they can also be extended to partial agonists.

Discussion

The concept that different degrees of intrinsic activity of agonist drugs should be observed in cells and tissues expressing different levels of a G protein-linked receptor is a classical element of receptor theory (1–3). Experimental examination of this concept traditionally has focused on the observations that many drugs act as full agonists in some tissues but not in others or has used irreversible antagonists to limit access of agonists to receptors. The first of these approaches is limited by the fact that levels of both the G protein(s) and the effector systems that are coupled to a particular receptor are likely to vary widely from cell type to cell type and are generally unknown, and the second is limited by the possibility that irreversible antagonism of a fraction of the receptors might be anticipated to limit access of the remaining receptors to a proportion of the G protein population. In cells in which G protein levels are comparable to those of the receptor, this might alter receptor output.

The availability of cDNA species encoding G protein-linked receptors has allowed the generation of clonal cell lines expressing varying receptor levels. Such an approach has been used previously to vary the level of β_2-adrenoceptors. Most recently, Whaley *et al.* (4) observed increased potency (decreased EC_{50}) of the natural ligand epinephrine with increas-

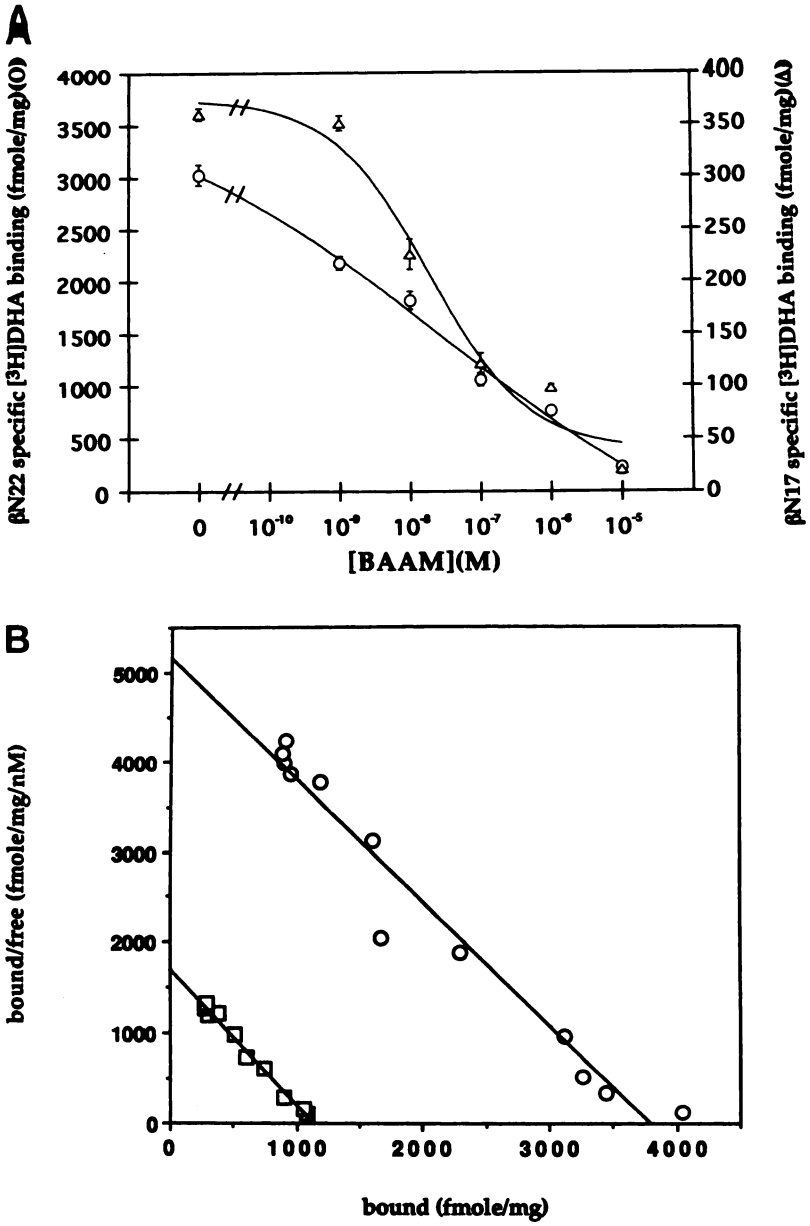

Fig. 3. Effect of BAAM treatment on specific binding of [^{3}H]DHA to membranes of βN22 and βN17 cells. A, BAAM pretreatment reduces the detected number of specific [^{3}H]DHA binding sites. Cells of clones βN22 and βN17 were treated in culture with varying concentrations of BAAM (up to 10 μM) for 4 hr. After cell harvest and membrane preparation, the specific binding of a single concentration of [^{3}H]DHA to available β_2-adrenoceptors was measured as described in Experimental Procedures. The results are the means ± standard deviations of two experiments. B, BAAM pretreatment reduces the maximal binding of [^{3}H]DHA without altering the affinity of the remaining receptors for [^{3}H]DHA. Cells of clone βN22 were not treated (○) or were treated with BAAM (100 nM) for 4 hr (□). Membranes were then prepared and saturation binding studies were performed with [^{3}H]DHA to determine both the maximal capacity and the affinity of this ligand for the available receptors. Data are presented as a Scatchard plot. In the example displayed, B_{max} was 3796 fmol/mg of membrane protein in the control membranes and 1142 fmol/mg of membrane protein in the BAAM-pretreated cells, whereas the K_d for [^{3}H]DHA was 0.74 nM in the controls and 0.67 nM after treatment with BAAM.

ing receptor number. We note a similar observation herein for full agonists and extend this relationship to partial agonists. Such a relationship was also observed by Bouvier *et al.* (5), as was an increasing measured intrinsic activity for isoprenaline with increasing receptor expression, which was, however, reduced markedly in cells expressing very high levels of the receptor. Following a different strategy, Johnson *et al.* (6) obtained β-adrenoceptor-deficient variant cells by clonal selection; those authors, however, did not observe any difference in agonist potency. Because such previous reports have varied somewhat in the parameters measured and in the results obtained, we wished to extend these studies. Furthermore, these previous studies have concerned themselves only with full β-adrenoceptor agonists.

In this study we have used a stable cDNA cellular expression approach in concert with the more classical use of irreversible receptor blockade to vary receptor availability to ligands. Importantly, given the points made above, for these studies we selected clonal cell lines after transfection of NG108–15 cells with the human β_2-adrenoceptor (7, 8), because we previously measured the number of copies per cell of the α subunit of the G protein G_s (1.25×10^6/cell) and the maximal number of copies of the complex of adenylyl cyclase and $G_{s\alpha}$ (i.e., the activated form of adenylyl cyclase) (1.7×10^4/cell), which can be formed by addition of a poorly hydrolyzed GTP analogue or NaF to membranes of these cells. Those studies showed the G protein to be in considerable molar excess (>70-fold) over adenylyl cyclase (9), and thus we know the stoichiometry of expression of each component of the signaling cascade that we have studied. We also previously noted that immunological levels of both $G_{s\alpha}$ (7) and adenylyl cyclase (10) are unaltered, compared with the parental cell line, in each of the β_2-adrenoceptor-transfected clones that we analyze herein.

Maximally effective concentrations of isoprenaline (up to 10 μM) resulted in similar adenylyl cyclase activities in membranes of clones βN22 and βN17. However, when a series of traditional β-adrenoceptor partial agonists were examined,

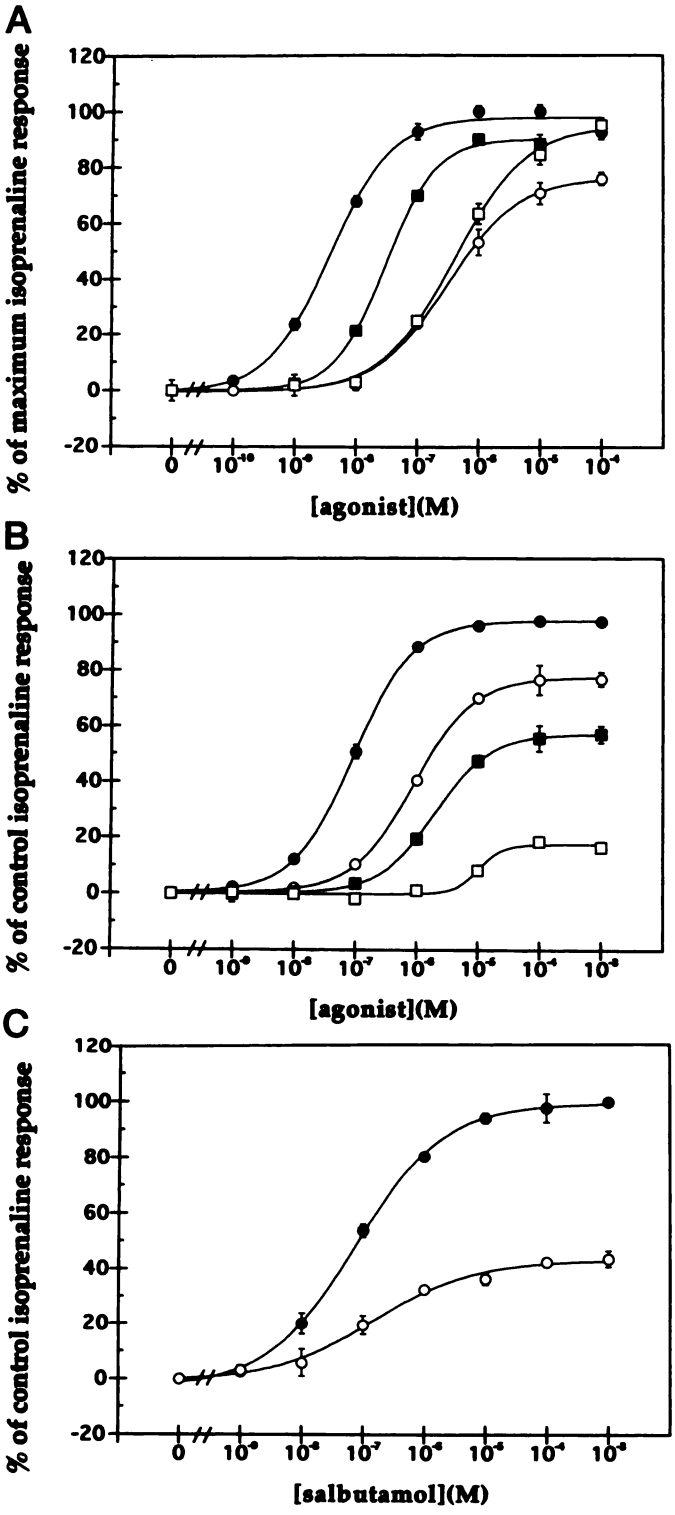

Fig. 4. Effect of BAAM pretreatment of βN22 cells on the potencies and measured intrinsic activities of β-adrenoceptor-active drugs. A and B, Membranes were prepared from βN22 cells that had been either not treated (*closed symbols*) or pretreated with BAAM (1 μM, or 10 μM in the studies with isoprenaline) for 4 hr (*open symbols*). Adenylyl cyclase activity was then assayed in the presence of varying concentrations of the full agonists isoprenaline (*circles*) or epinephrine (*squares*) (A) or the partial agonists salbutamol (*circles*) or ephedrine (*squares*) (B). Results are displayed as percentage of the effect obtained with isoprenaline in membranes of untreated βN22 cells. C, The ability of salbutamol to stimulate specific [^{3}H]forskolin binding in whole untreated (●) and BAAM (1 μM)-treated (○) βN22 cells was also assessed, and the results are displayed in the same fashion. In the experiments displayed, basal adenylyl cyclase activity in βN22 preparations was 62.4 ± 8.3 pmol/min/mg of membrane protein. Isoprenaline (10 μM)-stimulated adenylyl cyclase activity in βN22 preparations was 211.0 ± 8.1 pmol/min/mg of membrane protein (mean ± standard deviation, three experiments). Specific binding of [^{3}H]forskolin to βN22 cells and 1 μM BAAM-treated βN22 cells was 602 ± 89 dpm/10^5 cells and 565 ± 94 dpm/10^5 cells, respectively, in the presence of 10 μM isoprenaline (mean ± standard deviation, two experiments).

the measured intrinsic activities of these compounds, compared with isoprenaline, were routinely observed to be higher in membranes of clone βN22 cells than clone βN17 cells (Fig. 2). For example, salbutamol, which in membranes of clone βN17 cells was a partial agonist displaying an intrinsic activity of approximately 70%, compared with isoprenaline, acted as a full agonist in membranes of clone βN22 cells; ephedrine, with an intrinsic activity of approximately 20% in membranes of clone βN17 cells, was also considerably more efficacious in clone βN22 cell membranes (intrinsic activity of 65%). To extend the generation of primary data and the subsequent analysis in this study, we have made use of a second assay, in which agonist activation of the β_2-adrenoceptor on intact clone βN22 and βN17 cells causes an increase in high affinity [^{3}H]forskolin binding because the complex formed between $G_{s\alpha}$ and adenylyl cyclase is the high affinity binding site for this ligand (10, 15, 17). This assay was particularly attractive to us for this study because it allowed whole-cell analysis of measured intrinsic activities and thus a comparison with data generated using cell membranes. As with the adenylyl cyclase assay, maximally effective concentrations of isoprenaline (10 μM) resulted in similar levels of [^{3}H]forskolin binding to βN22 and βN17 cells and the measured intrinsic activities of the β-adrenoceptor partial agonists were again substantially higher in clone βN22 cells, compared with βN17 cells (Fig. 2B).

As noted above, considerable interest exists in the relationship between the measured EC_{50} for a drug response and the nature and level of receptor expression (4–6, 18, 19). Differences in EC_{50} values in whole tissues or in systems in which the receptor population for a ligand is poorly characterized are often interpreted to reflect receptor subtypes (19–21), although this would not be the likely conclusion if, for example, activation occurred via distinct G proteins or if the agonist structure-activity profile remained unaltered across a variety of chemical structures. Mathematical predictions have recently been developed to analyze alterations in β-adrenoceptor full agonist EC_{50} values with receptor number (4) and, in systems in which the effector species is quantitatively the limiting component of a signal transduction cascade, it is often observed that elevations in receptor number result in a leftward shift in the dose-effect curve (i.e., a reduction in the EC_{50} value), consistent with the notion of a receptor population reserve. We have previously recorded exactly this phenomenon when measuring isoprenaline stimulation of adenylyl cyclase activity in membranes of βN22 and βN17 cells (8), and a number of other reports have recorded similar data with cells expressing various levels of this receptor (4, 5), although one report in S49 lymphoma cells did not observe this effect (6). This is rather surprising, because S49 lymphoma cells are the one other cell system, apart from NG108–15 cells, in which the absolute number of copies of receptor, $G_{s\alpha}$, and adenylyl cyclase have been cal-

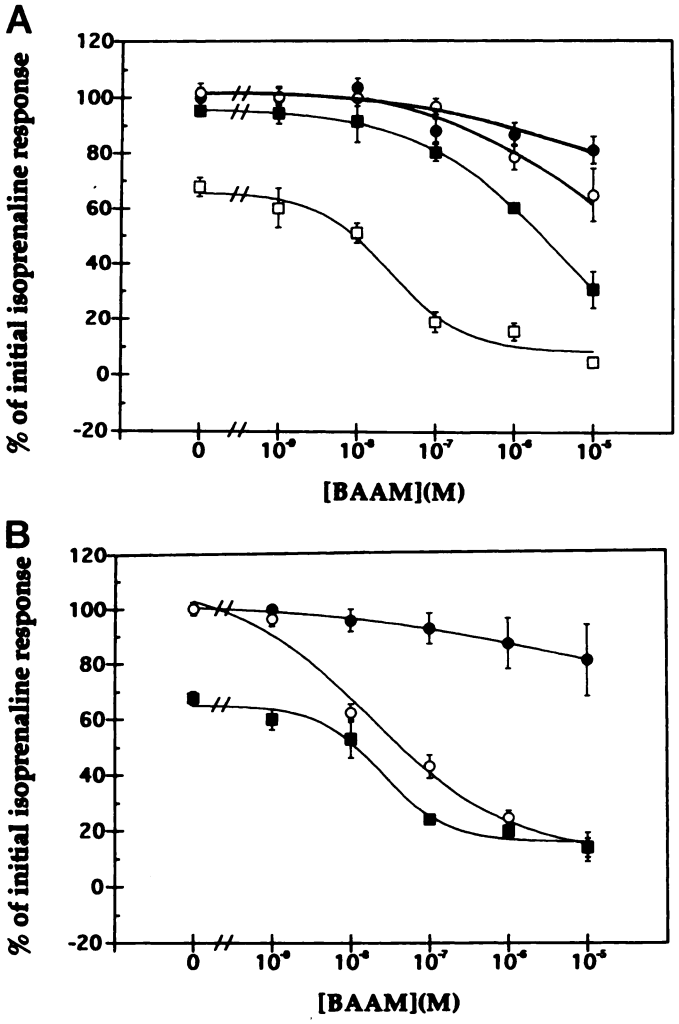

Fig. 5. Regulation by receptor availability of the measured intrinsic activity of β-adrenoceptor-active ligands in βN22 cells. A, Adenylyl cyclase assays. Membranes were prepared from clone βN22 cells that had been pretreated with varying concentrations of BAAM for 4 hr. Adenylyl cyclase activity and its regulation by maximally effective concentrations (10 μM) of isoprenaline (●), epinephrine (○), salbutamol (■), and ephedrine (□) were then assessed. Results are presented as percentage of the effect obtained in membranes of untreated cells with 10 μM isoprenaline. In the experiments displayed, basal adenylyl cyclase activities in βN22 and βN17 preparations were 61.4 ± 4.2 and 40.5 ± 5.2 pmol/min/mg of membrane protein, respectively. Isoprenaline (10 μM)-stimulated adenylyl cyclase activities in βN22 and βN17 preparations were 186.4 ± 6.4 and 136.3 ± 23.9 pmol/min/mg of membrane protein, respectively (mean ± standard deviation, two experiments). B, [^{3}H]Forskolin binding assays. The ability of maximally effective concentrations (10 μM) of isoprenaline (●), salbutamol (○), and ephedrine (■) to stimulate high affinity [^{3}H]forskolin binding in clone βN22 cells that had been pretreated with varying concentrations of BAAM for 4 hr was assessed. Results are presented as percentage of the effect produced in untreated cells by 10 μM isoprenaline. Control specific [^{3}H]forskolin binding represented 643 ± 103 dpm/10^5 cells (mean ± standard deviation, two experiments).

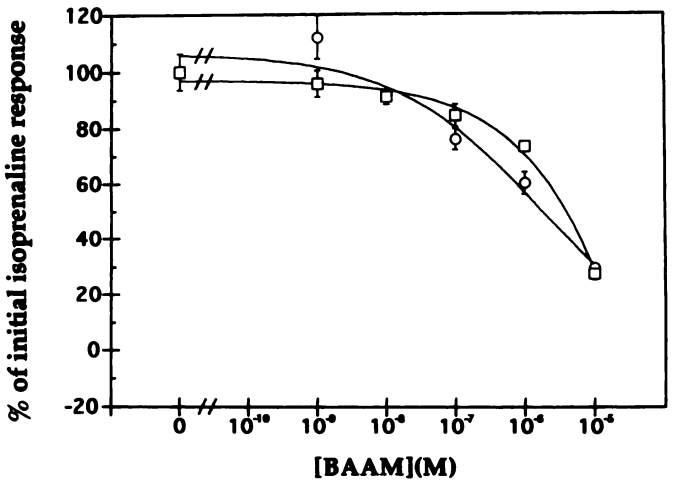

Fig. 6. Regulation by receptor availability of the measured intrinsic activity of isoprenaline in βN17 cells. Clone βN17 cells were pretreated with varying concentrations of BAAM for 4 hr. Either these were used directly in [^{3}H]forskolin binding assays (□) or membranes were prepared from these cells and adenylyl cyclase activity was measured (○). The effect of a maximally effective concentration of isoprenaline (10 μM) was assessed in both of these assays. Results are presented as percentage of the effect obtained in membranes of untreated cells with 10 μM isoprenaline. The results are the means ± standard errors of triplicate determinations.

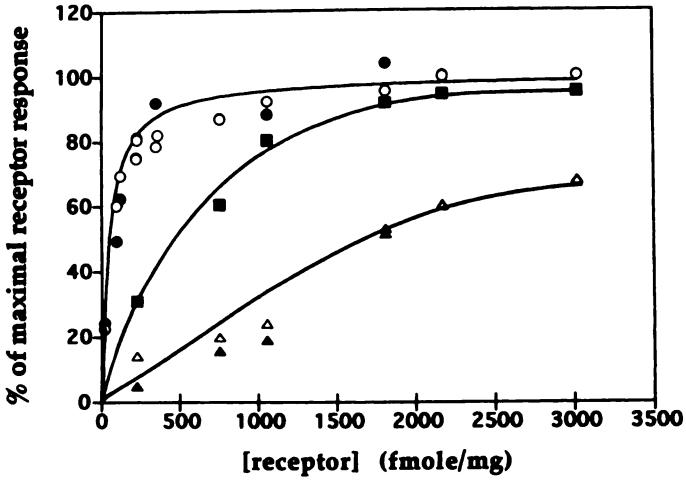

Fig. 7. Analysis of receptor levels in defining the observed intrinsic activities of β-adrenergic ligands. Adenylyl cyclase activity (*closed symbols*) and agonist regulation of [^{3}H]forskolin binding (*open symbols*) were measured in the presence of 10 μM isoprenaline (*circles*), salbutamol (*squares*), or ephedrine (*triangles*), in membranes (adenylyl cyclase activity) or whole cells ([^{3}H]forskolin binding) of clones βN22 and βN17 that either had not been treated or had been exposed to varying concentrations of BAAM, as described in Experimental Procedures. Data are presented for each ligand as the percentage of the maximal response that could be achieved in this genetic background (10 μM isoprenaline with untreated βN22 cells and membranes) versus the number of β_2-adrenoceptors available to be occupied.

culated (15) and, as in NG108–15 cells (9), a large molar excess of $G_{s\alpha}$ over adenylyl cyclase has been observed. It may be that in this system the differences in the levels of receptor expression in the individual clones were insufficient to allow detection of the effect.

The standard approach to altering available receptor levels in a tissue is to treat the tissue with varying concentrations of an irreversible antagonist for that receptor. When we treated βN22 cells with the irreversible β-adrenoceptor antagonist BAAM (1 μM, for 4 hr), a treatment sufficient to prevent binding of [^{3}H]DHA to approximately 75% of the initial receptor population (Fig. 3), the intrinsic activities and EC_{50} values of ephedrine and salbutamol were changed to levels close to those observed in untreated βN17 cells (compare Figs. 2 and 4). The relationship of intrinsic activity of partial agonists to receptor number was examined in detail

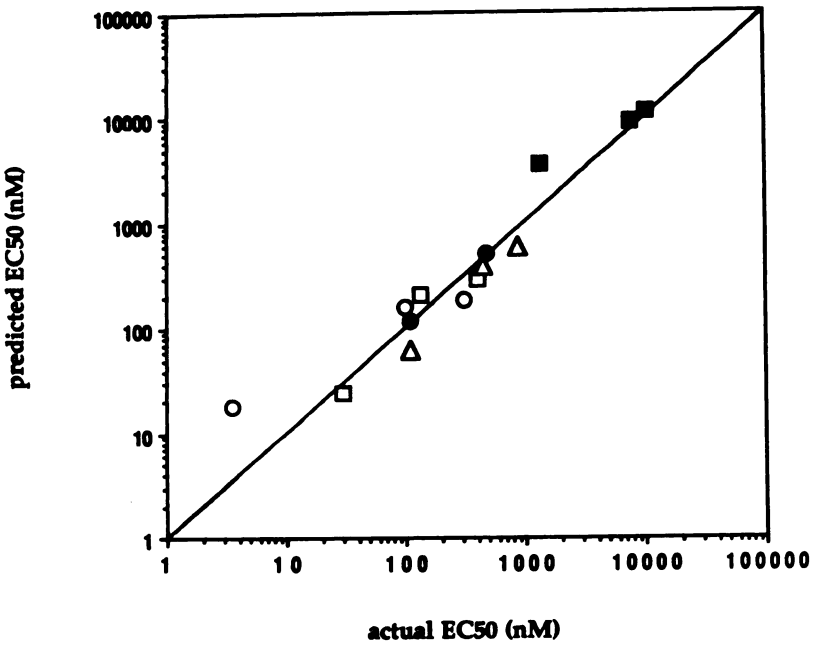

Fig. 8. Similarity between the actual experimental values for full agonist and partial agonist potencies and those theoretically predicted at various receptor levels. Comparison between the experimentally determined values and the theoretically predicted values for the potencies (EC_{50}) of isoprenaline (○), epinephrine (□), salbutamol (△), dobutamine (●), and ephedrine (■) under conditions of different receptor levels (comparing βN22, βN17, and BAAM-treated βN22 membranes) is shown. Calculated values were determined from adenylyl cyclase assays, as described in Results. For reasons of clarity, values are plotted on logarithmic scales and error bars are omitted. The *straight line*, with a slope of 1, is that which would indicate a perfect correlation between theoretical and actual data.

using membranes and cells treated with varying levels of BAAM, and noticeably and interestingly the measured regulation of agonist intrinsic activity was reduced by BAAM with greater potency the lower the measured intrinsic efficacy of the ligand in the untreated cells (Fig. 5). As such, the measured intrinsic activity of ephedrine in membranes and cells of clone βN22 was reduced to half-maximal levels by treatment with approximately 25 nM BAAM, a concentration sufficient to eliminate 50% of the receptor population, whereas this reduction was not achieved for salbutamol until approximately 75% of the receptors were irreversibly blocked (i.e., by treatment with 1.5 μM BAAM) (Fig. 5A). We were unable to use sufficiently high concentrations of BAAM to substantially reduce the measured intrinsic activity of isoprenaline in βN22 cells and membranes (Fig. 5). However, as a means to address the question of how many receptors would need to be occupied by a full agonist to half-maximally activate the cellular adenylyl cyclase population, we treated cells of clone βN17 with a range of concentrations of BAAM and then assessed the intrinsic activity of isoprenaline (Fig. 5). We observed that 50% of the intrinsic activity of isoprenaline, compared with that in untreated cells, was produced by pretreatment of βN17 cells with 2 μM BAAM, a concentration consistent with access of isoprenaline to only approximately 50 fmol/mg of membrane protein of the β_2-adrenoceptor in both the whole cells and the membranes derived from them. Presentation of data from such experiments in a form that indicates the levels of receptor required to be occupied by each of isoprenaline, salbutamol, and ephedrine to obtain different degrees of intrinsic activity for these ligands (Fig. 7) allows direct comparison of the absolute intrinsic activities of these ligands. Approximately 35-fold higher levels of β_2-adrenoceptors had to be occupied with ephedrine than with isoprenaline to obtain 50% of the maximal activation of the adenylyl cyclase cascade, whether this was measured as adenylyl cyclase activity in membranes or as stimulation of [^{3}H]forskolin binding in whole cells (Fig. 7), whereas salbutamol required occupancy of only one third as many receptors as did ephedrine. Such data demonstrate why isoprenaline and salbutamol act as full agonists in cells and membranes of clone βN22, whereas isoprenaline but not salbutamol is a full agonist in clone βN17. It further explains why ephedrine is not a full agonist in either system. It must be anticipated, however, that it would become so in clonal cell lines of this genetic background that express even higher levels of this receptor, but this obviously must await direct demonstration.

Whaley *et al.* (4) derived theory-based mathematical relationships that estimate the effect that varying β_2-adrenoceptor levels would be anticipated to have on full agonist potency, and they compared these predictions with their experimental data. As observed in this report, Whaley *et al.* (4) both predicted and experimentally demonstrated increased potency for epinephrine with increasing receptor number. However, as in other previous investigations (5, 6), experimental analysis was restricted to full agonists. Here, in an attempt to extrapolate the theoretical implications and findings of Whaley *et al.* (4) to ligands that are partial agonists, we have applied their theoretical equation to our data. As seen in Fig. 8, the predicted theoretical potency values are similar to the actual observed potencies not only for full agonists (isoprenaline and epinephrine) but also for partial agonists (salbutamol, dobutamine, and ephedrine), both in the two cell lines we have examined and after irreversible agonist blockade of proportions of the receptors.

The data reported herein provide novel insights into receptor theory and predictions of the behavior of partial agonists. They further demonstrate that a number of theoretical postulates of receptor theory can be examined by use of a combination of molecular biological and pharmacological approaches.

Acknowledgments

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