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Inverse Agonist Activity of β-Adrenergic Antagonists

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SUMMARY

Agonist-independent properties of the human β_2-adrenergic receptor (β_2AR) were studied using the baculovirus expression system in Sf9 cells. In the absence of agonist but in the presence of GTP, membranes from cells expressing the β_2AR exhibited higher levels of cAMP production than did membranes from uninfected cells or from cells infected with wild-type baculovirus. The increase in cAMP production was proportional to the number of β_2AR expressed, up to 40 pmol/mg of membrane protein, and it could be inhibited in a dose-dependent manner by βAR antagonists. The increase and its reversal both were independent of the possible presence of contaminating catecholamines in the culture medium and thus appear to reflect spontaneous β_2AR activity and direct antagonist-receptor interactions, respectively. The maximal level of inhibition varied among the βAR ligands tested, to yield the following rank order of "inverse efficacy": timolol ≥ propranolol > alprenolol ≥ pindolol > labetalol > dichloroisoproterenol. The same rank order was observed using membranes prepared from Chinese hamster fibroblasts expressing β_2AR. The effect of timolol was partly blocked by labetalol and dichloroisoproterenol, in an apparently competitive manner. The intracellular cAMP content of Sf9 cells cultured in serum-free medium was also increased by the expression of β_2AR, and that increase was reversed by timolol and propranolol, consistent with observations in membrane preparations. The properties revealed by the expression of the β_2AR in Sf9 cells suggest two agonist-independent traits of G protein-linked receptors, i.e., 1) that unliganded receptors are able to activate G proteins both in membrane preparations and in whole cells and 2) that antagonists may mediate their effects not only by preventing the binding of agonists but also by decreasing the propensity of the receptor to assume an active state.

Antagonists inhibit the binding of agonists to receptors, and the physiological effects of the former usually are attributed to their ability to prevent activation of receptors by endogenous hormones and neurotransmitters. In general, it is thought that antagonists do not modulate the activity of unliganded receptors, but a growing body of evidence contradicts this notion. Antagonists have been reported to produce effects opposite to those of the corresponding agonists at both benzodiazepine receptors and G protein-linked receptors (reviewed in Refs. 1 and 2, respectively). The direct, agonist-independent modulation of receptor activity by antagonists has been referred to variously as inverse agonism (3), negative or reverse intrinsic activity (2, 4), and negative antagonism (5).

At the benzodiazepine receptor/γ-aminobutyric acid type A receptor complex, benzodiazepine agonists allosterically increase the affinity of γ-aminobutyric acid, whereas inverse agonists have the opposite effect (3). The biochemical properties of benzodiazepine agonists are reflected *in vivo* by anxiolytic and anticonvulsant effects. Inverse agonists, in contrast, tend to be anxiogenic or proconvulsant (1, 6). It has been proposed that the physiological effects of agonists and inverse agonists in this system are correlated with their ability to stabilize either the "active" or the "inactive" form of the receptor complex, respectively (3, 6).

Similarly, antagonists have been reported to produce effects opposite to those of agonists at G protein-linked receptors, with respect to both second messenger (7) and G protein regulation (4, 8, 9). However, these effects are not well characterized and have been reported in reconstituted or broken cell preparations but not in intact systems. Thus, in contrast to the benzodiazepine receptor, a physiological correlate to the biochemical effects of inverse agonists appears to be lacking, and the relevance of agonist-independent effects of antagonists at G protein-coupled receptors is unclear.

Presumably, inverse agonism requires that G protein-linked receptors can exhibit spontaneous, agonist-independent activity, and some evidence suggests that this can occur in intact systems (10). It has been suggested that the demonstration of inverse agonism at G protein-linked receptors in such systems would lend support to the hypothesis that hormone-independent effects contribute to the biological responses observed with antagonists (2).

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ABBREVIATIONS: AR, adrenergic receptor(s); ISA, intrinsic sympathomimetic activity; CYP, cyanopindolol; CHW, Chinese hamster fibroblast(s).

In the present study we show that the human β_2AR increases the production of cAMP both in Sf9 cells and in membranes derived from those cells, in an agonist-independent manner, and that this increase can be inhibited by βAR antagonists. We also show that similar effects occur in membranes derived from mammalian cells expressing β_2AR. These observations suggest that the mechanism of action of βAR antagonists *in vivo* may be, in addition to preventing hormone binding, to decrease the propensity of the receptor to assume a conformation that is required for the stimulation of cAMP production.

Materials and Methods

Experimental Procedures

Cell culture and infection with recombinant baculovirus. Sf9 cells were cultured at 27° in Grace's supplemented insect medium (GIBCO), to which were added 10% fetal bovine serum (P.A. Biologicals, Sydney, Australia), 0.1% Pluronic F-68 (GIBCO), 50 μg/ml gentamicin sulfate, and 2.5 μg/ml fungizone. For some experiments, serum was omitted from the culture medium for the final 18 hr of infection. For most experiments, cells were grown in 100-ml spinner flasks; in some cases, cells were grown as monolayers in 75-cm^2 flasks. Cells were infected with wild-type or recombinant baculovirus when they reached a density of 1.0–4.0 × 10^6 cells/ml and were harvested 48 hr after infection, unless indicated otherwise. Experiments were carried out either on preparations of washed membranes or on whole cells.

Preparation of washed membranes from Sf9 cells. Washed membranes were prepared essentially as described previously (11). Infected cells (10 or 20 ml) were washed twice with 2 volumes of ice-cold phosphate-buffered saline and lysed with a Polytron homogenizer (two 5-sec bursts) in 10 ml of cold buffer containing 5 mM Tris·HCl, pH 7.4, 2 mM EDTA, 10 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor, and 5 μg/ml leupeptin. Lysates were centrifuged at 500 × *g* for 5 min at 4°, and the pellet was resuspended in 4 ml of buffer, homogenized, and centrifuged again. The two supernatants were pooled and centrifuged at 45,000 × *g* for 20 min at 4°, and the pellet was washed twice in 10 ml of the same buffer. The membranes were resuspended in buffer containing 75 mM Tris·HCl, pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA, 10 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor, and 5 μg/ml leupeptin and were then used for adenylyl cyclase or binding assays. Protein content was determined according to the method of Bradford (12).

Culture of mammalian cells and preparation of membranes. CHW cells expressing β_2AR (4–5 pmol of receptor/mg of protein) were cultured and membranes were prepared as described previously (13).

Assay of adenylyl cyclase activity. In membranes, assays were carried out using 5–10 μg of protein with 0.12 mM ATP, 1–2 × 10^6 cpm/assay tube of [α-^{32}P]ATP, 0.10 mM cAMP, 53 μM GTP, 2.7 mM phospho(enol)pyruvate, 1.0 IU of myokinase, 0.2 IU of pyruvate kinase, 4 μg/ml benzamidine, 2 μg/ml soybean trypsin inhibitor, 2 μg/ml leupeptin, and varying concentrations of AR ligands, in a total volume of 50 μl. Samples were incubated at 37° for 15 or 30 min, and the reaction was terminated by addition of 1 ml of a cold solution containing 0.3 mM cAMP, 20,000 cpm of [^{3}H]cAMP, and 0.4 mM ATP. [^{32}P]cAMP was separated by chromatography using a Dowex gel, followed by aluminium oxide (14).

Assay of intracellular cAMP. Intracellular cAMP was determined by competition with [^{3}H]cAMP for a specific binding protein (15). Cells that had been infected for 48 hr with either wild-type or recombinant baculovirus encoding the human β_2AR were deprived of serum for the final 18 hr of infection and then used for experiments. One-milliliter aliquots containing 4 × 10^6 to 1 × 10^7 cells were treated for 30 min at ambient temperature with various ligands in serum-free medium. cAMP was recovered from the cells as described previously (16) (Amersham kit no. TRK.432). One sample was prepared for each experimental condition, and each was assayed in duplicate for cAMP. Cellular cAMP was estimated by combining 50 μl of supernatant with [^{3}H]cAMP (4.5 nM final concentration) and allowing the labeled and unlabeled forms of the nucleotide to compete for a high affinity cAMP-binding protein for 2 hr at 4°; unbound [^{3}H]cAMP was removed by adsorption to charcoal followed by centrifugation, and the amount of radioactivity from the supernatant was determined. The amount of cAMP in each aliquot was estimated from a standard curve determined with samples that ranged from 1 to 16 pmol of cAMP/assay tube.

Binding of AR ligands. Washed membranes were labeled with ^{125}I-CYP to study the binding properties of the β_2AR. For determinations of capacity, membranes were incubated with a saturating concentration of the radioligand (≥300 pM; K_d = 33 pM) in the absence or presence of 10^{-5} M alprenolol, with the difference in bound ^{125}I-CYP between the two conditions being taken as the total specific binding. The inhibition of binding of a subsaturating concentration of ^{125}I-CYP (169 ± 8 pM) by increasing concentrations of unlabeled AR ligands was used to estimate the binding affinities of the latter compounds. In each case, assays were carried out in triplicate and membranes were incubated for 1.5 hr at ambient temperature in buffer containing 75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, 5 mM EDTA, 10 μg/ml benzamidine, 5 μg/ml soybean trypsin inhibitor, and 5 μg/ml leupeptin. Unbound radioligand was removed by rapid filtration through Whatman GF/C filters, which were then rinsed three times with 2 ml of 25 mM Tris, pH 7.4, at 4°.

Analysis of Results

The concentration dependence of adenylyl cyclase activity on various AR ligands in membranes was analyzed according to a four-parameter logistic equation analagous to the Hill equation (ALLFIT) (17). For all analyses, the slope factor was fixed at 1. For each βAR ligand listed in Table 1, replicate experiments were fitted simultaneously with EC_{50} common to all sets of data, allowing the asymptotes (i.e., $y_{x=0}$ and $y_{x=\infty}$) to vary for individual sets of data. The fitted values of $y_{x=0}$ and $y_{x=\infty}$ were used to define a_S and a_I, respectively, for the calculation of E_{inv} (see below). The inhibition of ^{125}I-CYP binding by βAR antagonists was analyzed according to the multisite model by nonlinear least-squares regression, as described (18). Data were analyzed assuming a single class of binding sites. For each unlabeled ligand, data from all experiments were fitted simultaneously with the affinity (K_d) common to all sets of data. Estimates of nonspecific binding and total receptor concentration were allowed to vary among individual sets of data. Unless specified otherwise, values listed in the text, table, and figure legends represent averages ± standard errors, and such values were compared where appropriate using Student's *t* test; for fitted values of K_d, EC_{50}, $y_{x=\infty}$, and $y_{x=0}$, the accompanying error reflects the range over which the sum of squares of the residuals is insensitive to the value of the parameter.

Inverse efficacy (E_{inv}) is defined as the fraction of spontaneous (i.e, agonist-independent) receptor activity that can be inhibited by a given agent, as shown in the equation $E_{inv} = A_I/A_R$, where $A_I = (a_S - a_I)/a_S$ and $A_R = (a_S - a_0)/a_S$. A_R represents the relative signal that is attributable to the receptor alone, i.e., the difference between the signal observed in the presence of receptor but in the absence of agonist or antagonist (a_S) and that observed in the absence of receptor (a_0), divided by a_S. A_I represents the relative decrease induced by maximal concentrations of a given agent, i.e., the difference between a_S and the signal observed at a saturating concentration of ligand (a_I), divided by a_S.

In the present study, a_S and a_I represent cAMP production in the absence of AR ligand and in the presence of a maximally effective concentration of the latter, respectively. These were estimated from simultaneous analyses, as described above, for experiments carried out in membranes; for experiments carried out in whole cells the measured values were used. The parameter a_0 represents the rate of cAMP production observed with preparations lacking β_2AR in the absence of any AR ligand. The mean values of A_R were 0.78 ± 0.02 in membranes (11 experiments) and 0.64 ± 0.17 in whole cells (four experiments), and

these were taken as constants in further calculations. For both membrane and whole-cell assays, values of A_I for individual ligands were averaged, and each value of E_{inv} was calculated by dividing the mean value of A_I by the mean value of A_R.

Results

Effect of human βAR expression on the production of cAMP. In washed membranes prepared from Sf9 cells, the level of cAMP produced in the presence of 53 μM GTP was found to be greater with cells infected for 48 hr with baculovirus encoding the human β_2AR than with either cells infected with wild-type baculovirus or uninfected cells (Fig. 1). To establish that the increase in cAMP was not due to stimulation of the receptor by contaminating catecholamines that may have been present in the complete medium, experiments were carried out on cells maintained in serum-free medium for the final 18 hr and treated with alprenolol (1 μM) for the final 30 min of the infection. As shown in Fig. 1, *inset*, the receptor-mediated increase was still observed under these conditions. An increase was also observed with serum-deprived cells that had not been treated with alprenolol (data not shown). It follows that the β_2AR is spontaneously active in washed membranes. In addition to the receptor-mediated, agonist-independent increase in adenylyl cyclase activity observed in membranes from cells expressing β_2AR, the βAR agonist isoproterenol further stimulated the enzyme. The agonist was without effect on membranes from cells that did not express the receptor (Fig. 1).

Infections for 48 hr at various multiplicities of infection yielded levels of β_2AR expression ranging from 0.05 to 45 pmol/mg of protein. As shown in Fig. 2, the agonist-independent and the net isoproterenol-stimulated and forskolin-stimulated increases in cAMP production all were linearly correlated with the level of β_2AR expression ($r^2 = 0.97$, $r^2 = 0.99$, and $r^2 = 0.98$, respectively).

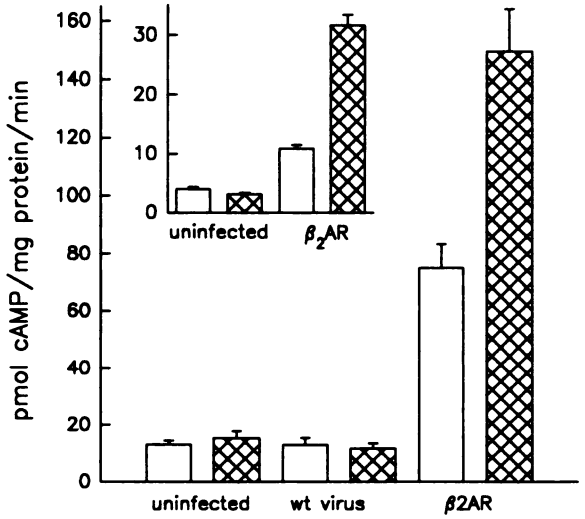

Fig. 1. Activity of adenylyl cyclase in membranes from uninfected Sf9 cells and Sf9 cells infected for 48 hr with either wild-type (*wt*) baculovirus or recombinant baculovirus encoding the human β_2AR. The production of cAMP was measured in the absence (□) or presence of 10 μM isoproterenol (■). The data shown represent means from at least three experiments carried out in duplicate. *Inset*, the production of cAMP was measured in membranes from cells that had been deprived of serum for the final 18 hr of infection and treated with 1 μM alprenolol for 30 min before harvesting.

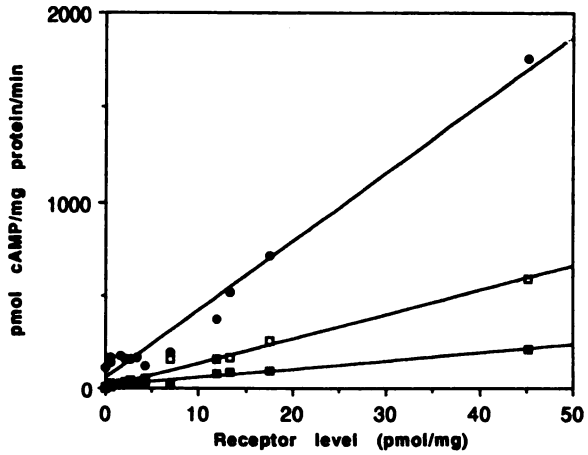

Fig. 2. Effect of receptor level on GTP-, isoproterenol-, and forskolin-stimulated adenylyl cyclase activity. The level of β_2AR in membranes was varied by infecting Sf9 cells for 48 hr at increasing multiplicities of infection, and receptor levels were determined as described in Materials and Methods. Production of cAMP was measured in the presence of 53 μM GTP alone (■) or with the additional inclusion of 10 μM isoproterenol or 100 μM forskolin. The net stimulation by isoproterenol (□) and forskolin (●) was determined by subtracting the level of activity observed in the presence of GTP only, at each concentration of receptor.

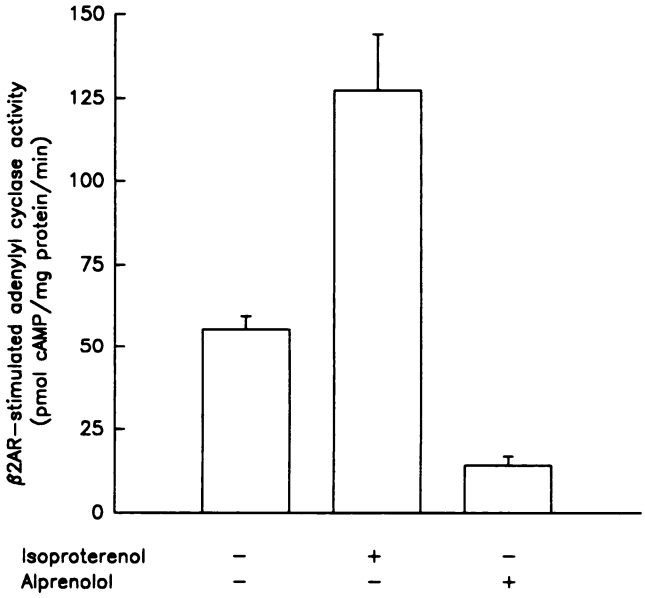

Fig. 3. Effect of alprenolol on β_2AR-stimulated adenylyl cyclase activity. The production of cAMP in membranes from Sf9 cells lacking β_2AR (i.e., either uninfected or infected with wild-type baculovirus) was subtracted from that in membranes expressing the receptor, in the presence of isoproterenol (10 μM), alprenolol (10 μM), or neither. The data shown represent means from four experiments.

Inhibition of receptor-stimulated cAMP production. Alprenolol partly reversed the increase in cAMP production associated with the expression of the β_2AR, whereas isoproterenol stimulated adenylyl cyclase activity >2-fold in the same preparations (Fig. 3). When membranes containing β_2AR were treated with both isoproterenol and alprenolol, a net decrease was observed in the level of enzymic activity (data not shown), similar to that found with alprenolol alone. Membranes from cells lacking the receptor exhibited similar levels of enzymic activity in the presence and absence of alprenolol ($p = 0.82$), indicating that the inhibition was due to an interaction between

alprenolol and the receptor. To ensure that competition with serum catecholamines in the culture medium was not responsible for the observed inhibition, experiments were carried out on cells maintained in serum-free medium. Alprenolol was found to inhibit adenylyl cyclase in a dose-dependent manner in washed membranes from cells cultured under such conditions (Fig. 4). This inhibition was observed even when cells were treated with alprenolol for 30 min before harvesting. Similarly, treatment of cells maintained in complete medium with 1 μM alprenolol for 30 min before harvesting of membranes did not prevent the decrease in cAMP production with alprenolol (data not shown), indicating that the inhibition observed under normal experimental conditions was not dependent upon contaminants in the medium.

Propranolol also was found to inhibit the β_2AR-associated increase in adenylyl cyclase activity (Fig. 5). The magnitude of the response appeared to be limited by the receptor-induced increase in cAMP. In absolute terms, the inhibition of the enzyme by propranolol (Fig. 5) or alprenolol (data not shown) increased with the receptor-dependent increase in cAMP. At the lowest concentrations of receptor investigated (12-hr infection, 0.03 ± 0.02 pmol of β_2AR/mg of protein), the inhibition could not be observed reliably, although a small amount of stimulation was observed under such conditions in response to isoproterenol (data not shown). The maximal levels of both the stimulation and the inhibition of adenylyl cyclase thus appear to be dependent upon the density of β_2AR in the membrane.

A variety of AR ligands were characterized with respect to agonist-independent inhibitory potency, binding affinity, and inverse efficacy (Fig. 6; Table 1). The production of cAMP in

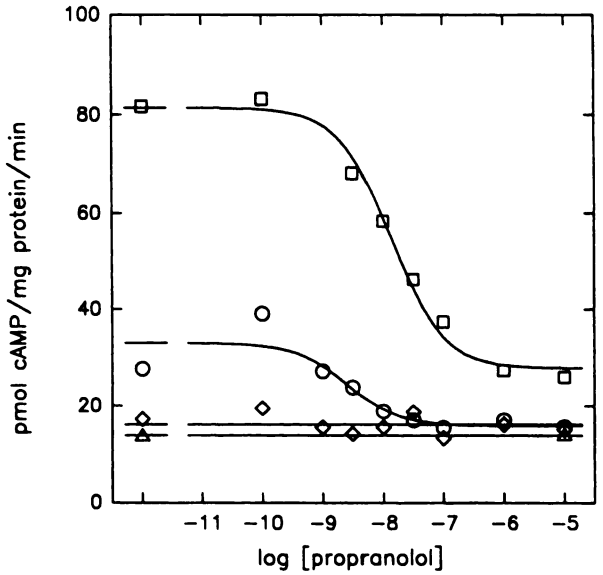

Fig. 5. Effect of time of infection on the inhibition of membrane adenylyl cyclase activity by propranolol. Sf9 cells were infected with baculovirus encoding the β_2AR for 0 (△), 12 (◇), 24 (○), or 48 hr (□) before preparation of membranes, and the production of cAMP was measured at the concentrations of propranolol indicated on the *abscissa*. The estimated membrane concentrations of β_2AR in this experiment were 0.04, 1.9, and 10.0 pmol/mg of protein at 12, 24, and 48 hr of infection, respectively. The data shown are representative of two experiments, and a comparable pattern was observed with alprenolol.

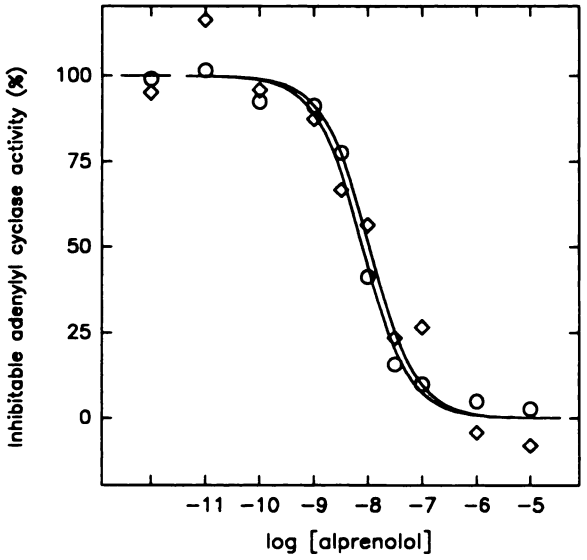

Fig. 4. Inhibition of agonist-independent β_2AR-stimulated adenylyl cyclase by alprenolol in membranes from cells deprived of serum. Sf9 cells infected with baculovirus and cultured in serum-free medium for the final 18 hr of infection were treated with vehicle (○) or 1 μM alprenolol (◇) for 30 min before membrane preparation. The production of cAMP was measured at the concentrations of alprenolol shown on the *abscissa*. The data shown are from a representative experiment (two determinations) and were normalized to the fitted asymptotes, which were as follows: estimated cAMP production in the absence of alprenolol ($y_{x=0}$) was 45 ± 1 and 75 ± 1 pmol/mg/min in alprenolol-treated and control membranes, respectively, and that in the presence of a maximally inhibiting amount of alprenolol ($y_{x=\infty}$) was 30 ± 1 and 34 ± 1 pmol/mg/min in alprenolol-treated and control membranes, respectively.

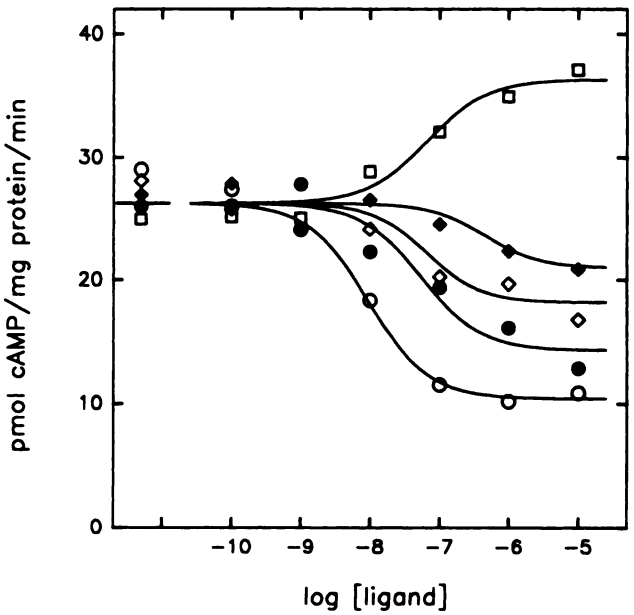

Fig. 6. Inhibition of agonist-independent adenylyl cyclase activity in membranes from Sf9 cells expressing β2AR. Production of cAMP was measured in the presence of dichloroisoproterenol (◆), labetalol (◇), pindolol (●), or timolol (○), at the concentrations indicated on the *abscissa*. The representative data shown were obtained on the same day with the same membrane preparation and were included in the simultaneous analyses for the determination of the EC_{50} and E_{inv} values listed in Table 1. Isoproterenol (□) was included as a positive control. For the curves shown, the data were reanalyzed with $y_{x=0}$ common to all five sets of data.

TABLE 1

Inhibition of β_2AR-associated increase in membrane adenylyl cyclase activity by AR ligands

Sf9 cells were infected for 48 hr with baculovirus encoding the β_2AR, to yield 23 ± 2 pmol of receptor/mg of membrane protein.

βAR ligands	EC_{50}[a]	n[b]	K_d[a]	n	E_{inv}[c]	n
	nM		nM			
Alprenolol	6.7 ± 1.1	6	0.32 ± 0.06	3	0.68 ± 0.02	6
Propranolol	7.9 ± 1.2	4	0.36 ± 0.08	3	0.82 ± 0.04	4
Timolol	10.0 ± 1.8	6	0.29 ± 0.07	2	0.91 ± 0.04	6
Pindolol	36 ± 8	6	0.71 ± 0.01	3	0.65 ± 0.04	6
Labetalol	61 ± 44	4	10.6 ± 2.8	2	0.40 ± 0.03	4
Dichloroisoproterenol	760 ± 396	4	220 ± 92	1	0.09 ± 0.11	4
αAR ligands			K_d[d]	n	E_{inv}	n
			μM			
Prazosin	e		2.3 ± 1.1	2	≥0.7	3
Phentolamine	e		31 ± 16	2	≥0.5	3
Yohimbine	e		592 ± 83	2	≥0.5	3

[a] Errors accompanying EC_{50} and K_d values reflect the range over which the sum of squares is insensitive to the value of the parameter.
[b] n, number of experiments.
[c] Values are mean ± standard error.
[d] Specific binding was not completely inhibited at the highest concentrations of these ligands used (2.0 mM) and nonspecific binding was set equal to that estimated from concurrent experiments with βAR antagonists.
[e] Maximal effect was not defined at the highest concentrations tested (0.1–1.0 mM), and therefore EC_{50} could not be estimated reliably.

membranes from cells expressing β_2AR was increased 4.5-fold on average, relative to that in membranes from cells lacking the receptor; none of the agents tested decreased cAMP production to a level below that observed in membranes from cells lacking β_2AR. The βAR ligands tested varied considerably with respect to their effects on adenylyl cyclase activity, as illustrated in Fig. 6. Timolol caused the greatest inhibition and dichloroisoproterenol the least. Isoproterenol typically stimulated enzymic activity 1.5–2.5-fold.

The inhibitory effects of nine different AR ligands on the binding of ^{125}I-CYP and on the production of cAMP in membranes from β_2AR-expressing Sf9 cells are summarized in Table 1. In general, the rank orders of affinity (K_d) and potency (EC_{50}) are in agreement with each other. For each inhibitory βAR ligand tested, the EC_{50} value exceeded the K_d value (Table 1).

There was no obvious relationship between the inverse efficacy of a given ligand and its EC_{50}, K_d, or EC_{50}/K_d value. If the observed inhibition were due to competition between the ligands tested and contaminating catecholamines, a correlation between E_{inv} and K_d would be expected. Timolol, propranolol, and alprenolol all yielded similar values with respect to potency and binding affinity, but the inverse efficacy of alprenolol was significantly less than that of either timolol ($p = 0.002$) or propranolol ($p = 0.032$). Pindolol was less potent than any of those three agents but exhibited an inverse efficacy similar to that of alprenolol. Of the βAR ligands tested, dichloroisoproterenol was the weakest with respect to potency, affinity, and inverse efficacy.

Three αAR antagonists, i.e., phentolamine, prazosin, and yohimbine, were also tested. Although they bound with affinities several orders of magnitude weaker than those of most of the βAR antagonists tested, these agents inhibited β_2AR-induced cAMP production by at least 50%, further demonstrating the lack of corrlation between E_{inv} and K_d. For example, the inverse efficacy of yohimbine was greater than that of labetalol, even though the K_d of the former was approximately 4 orders of magnitude higher than that of the latter. Precise values for EC_{50} and E_{inv} could not be reliably determined for the αAR antagonists, because enzymic activity was still decreasing at the highest concentrations of those ligands tested (≥100 μM) and $y_{x\to\infty}$ was therefore undefined. Although an analogous problem was encountered in the binding experiments, values of K_d could be estimated by assuming that the nonspecific binding of ^{125}I-CYP was the same with both βAR and αAR ligands. Comparison of values of K_d with the incomplete dose-response curves obtained with prazosin, phentolamine, and yohimbine (data not shown) suggests that the rank orders of affinity and potency are the same for αAR antagonists, consistent with the effects of βAR ligands.

Competitive effects among ligands with different inverse efficacies. The agonist-independent inhibition of adenylyl cyclase activity by timolol could be partly blocked by less efficacious agents. At 100 nM timolol, the inhibition of cAMP production was nearly maximal (see Fig. 6), but the additional inclusion of labetalol (100 μM) partially reversed that inhibition (Fig. 7A). Similarly, higher concentrations of timolol were required to inhibit cAMP production in the presence of dichloroisoproterenol (100 μM) (Fig. 7B). It is clear that dichloroisoproterenol was not acting as a partial agonist, because it reduced the increase in enzymic activity attributable to the spontaneous activity of the receptor. The inhibition of the effect of timolol by ligands with relatively low inverse efficacies suggests that the different antagonists compete for a common binding site and that the incomplete effects of ligands such as labetalol do not arise from a failure to saturate the receptor. The latter point is supported by the observation that both labetalol and dichloroisoproterenol fully inhibit the specific binding of ^{125}I-CYP (Table 1).

Production of cAMP in whole cells. The expression of β_2AR was found to be associated with a 2.8-fold increase in intracellular cAMP in Sf9 cells cultured in serum-free medium (Fig. 8), and a similar increase was observed in cells cultured in complete medium (data not shown), which is consistent with observations in washed membranes. This suggests that the increase in adenylyl cyclase activity observed in membranes reflects the situation in whole cells. Moreover, the agonist-independent increase in cAMP in the absence of serum in Sf9 cells expressing the β_2AR was inhibited by the βAR antagonists propranolol and timolol. In contrast to their inhibitory effects

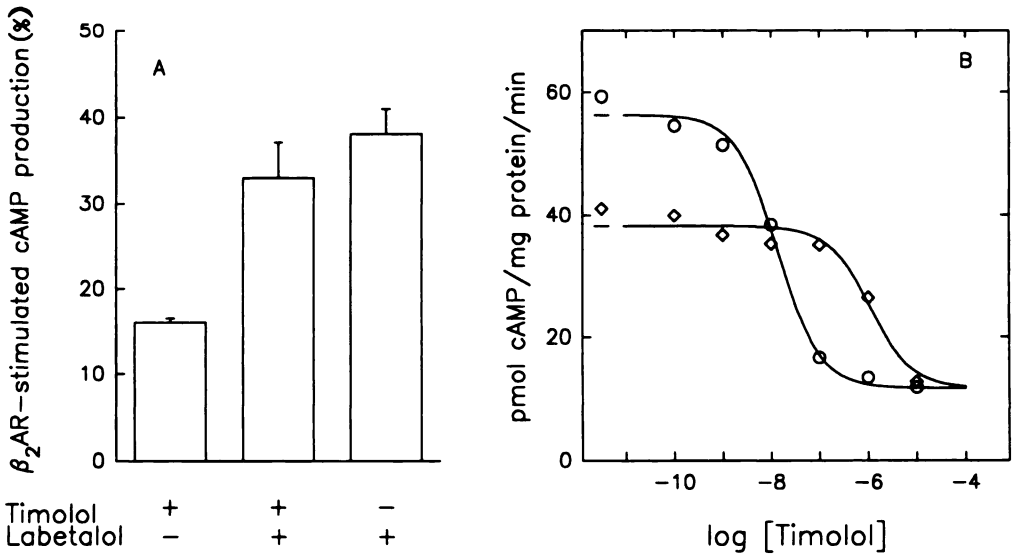

Fig. 7. Effect of agents with low E_{inv} on inhibition of adenylyl cyclase activity by timolol. A, Proportion of adenylyl cyclase activity associated with β_2AR expression observed in the presence of 10 nM timolol, 10 μM labetalol, or both. The results shown represent means from two experiments. B, Effect of timolol on the production of cAMP in membranes from Sf9 cells expressing β_2AR, in the absence (O) and presence (◇) of 10 μM dichloroisoproterenol. The data shown are representative of two experiments.

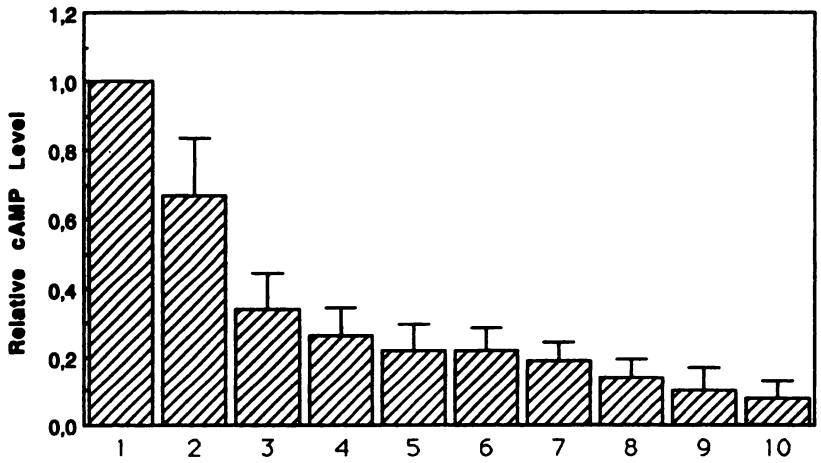

Fig. 8. Effect of βAR ligands on intracellular levels of cAMP in Sf9 cells expressing β_2AR. Cells were treated for 30 min before assay of intracellular cAMP concentrations. The data from each experiment were scaled by dividing the level of intracellular cAMP observed under each condition by that observed in the presence of 1 μM isoproterenol in the same experiment (the average of the latter from four experiments was 17.1 ± 6.6 pmol/sample), and the data shown represent the means of two to four independent observations. *Error bars*, standard deviations. *1-9*, Sf9 cells infected with recombinant baculovirus encoding the β_2AR; *10*, Sf9 cells infected with wild-type baculovirus. Treatments were as follows: *1*, 1 μM isoproterenol (four experiments); *2*, 10 μM dichloroisoproterenol (three experiments); *3*, 10 μM labetalol (two experiments); *4*, 10 μM pindolol (three experiments); *5*, no treatment (four experiments); *6*, 10 μM alprenolol (four experiments); *7*, 1 μM isoproterenol plus 10 μM alprenolol (four experiments); *8*, 10 μM propranolol (four experiments); *9*, 10 μM timolol (three experiments); *10*, no treatment (three experiments).

on membranes, alprenolol and pindolol had minimal effects on intracellular cAMP. Alprenolol was slightly inhibitory, and pindolol was slightly stimulatory. Furthermore, both labetalol and dichloroisoproterenol, which were relatively poor inhibitors in membranes, were clearly stimulatory in whole cells.

Surprisingly, the present results suggest that the same ligand can stimulate the activity of a receptor under one set of experimental conditions and inhibit the same receptor under a different set of conditions. Still, the ligands tested revealed the same rank order with respect to their effects in whole cells and in membranes. Moreover, although values of E_{inv} in whole cells were found to be negative for three of the six ligands (stimulatory ligands by definition have negative E_{inv} values), a significant correlation was found with E_{inv} in membranes ($r^2 = 0.94$) (Fig. 9). It follows that the phenomena observed in whole cells and in membranes may reflect the same underlying mechanisms.

Effect of forskolin on cAMP production and inverse agonism. The apparent loss of inverse agonism in whole cells with some of the ligands tested suggests that the "basal" activity of adenylyl cyclase may have differed between the two preparations tested. It follows that inverse agonism may be dependent upon not only the activation state of the receptor but also that of the effector. Experiments therefore were carried out to determine whether activation of adenylyl cyclase by forskolin would alter the βAR responses observed in Sf9 cells expressing the β_2AR. Adenylyl cyclase activity was stimulated by forskolin (1 μM) in Sf9 cells expressing β_2AR and in membranes derived

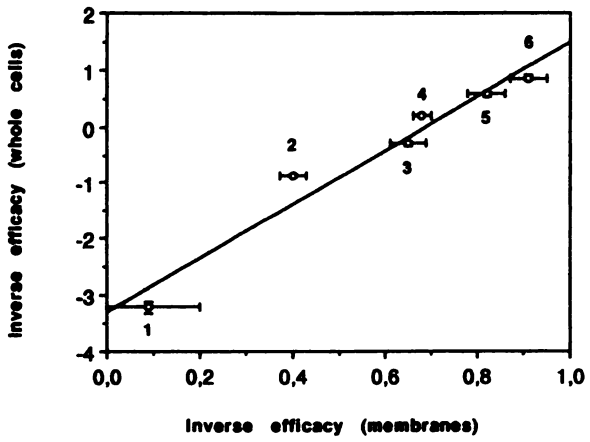

Fig. 9. Correlation between values of E_{inv} obtained in membranes (Table 1) and whole cells (Fig. 8) for the following ligands: *1*, dichloroisoproterenol; *2*, labetalol; *3*, pindolol; *4*, alprenolol; *5*, propranolol; *6*, timolol.

from those cells. Analogously to observations in the absence of forskolin, expression of the β_2AR was associated with increases in both intracellular cAMP and membrane adenylyl cyclase activity in the presence of forskolin (Fig. 10). Similarly, the effects of pindolol and propranolol were comparable in assays carried out in the presence and absence of forskolin; in both cases, pindolol was inhibitory in membranes (Figs. 6 and 10A) and slightly stimulatory in whole cells (Figs. 8 and 10B), whereas propranolol was inhibitory in membranes (Figs. 5 and 10A) as well as in whole cells (Figs. 8 and 10B). Forskolin thus appeared to have little influence on the overall effects of either the unliganded β_2AR or the inverse agonists tested on adenylyl cyclase activity in Sf9 cells.

Inhibition of receptor-stimulated cAMP production in mammalian cells. To ascertain whether the inverse agonist effects observed in Sf9 cells are also characteristic of β_2AR expressed in mammalian systems, experiments were carried out using membranes from CHW cells expressing human β_2AR. Agonist-independent adenylyl cyclase activity was inhibited in a dose-dependent manner by labetalol, pindolol, alprenolol, propranolol, and timolol (Fig. 11). The observed rank order of these ligands with respect to potency in CHW cells is the same as that obtained using the baculovirus/Sf9 expression system, as is the rank order with respect to inverse efficacy (Table 1).

Discussion

The results of the present study show that the agonist-independent, spontaneous activity of a G protein-coupled receptor in intact cells can be inhibited by antagonists (Fig. 8), suggesting that the pharmacological effects of the latter may arise at least partly from their direct interaction with receptors. The βAR ligands tested inhibited adenylyl cyclase in membranes to varying extents, as indicated by the range of E_{inv} values observed. As discussed below, it appears that the E_{inv} of a drug determined in membranes may reflect its pharmacological properties *in vivo*.

One potential caveat in studying the intrinsic effects of antagonists is that contaminating catecholamines may have been present in the serum included in the cell culture medium (19), but several observations indicate that both the β_2AR-induced increase in adenylyl cyclase activity and the inhibition of that activity by antagonists are independent of the possible influence of such contaminants. 1) It is estimated that the soluble constituents in the culture medium were diluted at least 10^7-fold during the routine preparation of the washed membranes. 2) Both the increase in adenylyl cyclase activity (Fig. 1, *inset*, and Fig. 8) and the inhibition of the enzyme by antagonists were observed in the absence of serum (Figs. 4 and 8). 3) Alprenolol inhibited the enzyme in membranes prepared from cells that had been cultured in the absence of serum and/or exposed to alprenolol before harvesting (Fig. 4). 4) E_{inv} was not correlated with binding affinity (Table 1), a relationship that would be expected if residual catecholamines were responsible for the increase in adenylyl cyclase activity. 5) The effect of timolol was inhibited both by labetalol and by dichloroisoproterenol (Fig. 7); if inhibition by timolol were due to the displacement of bound activating ligand, an additional inhibitory ligand would not be expected to decrease that effect.

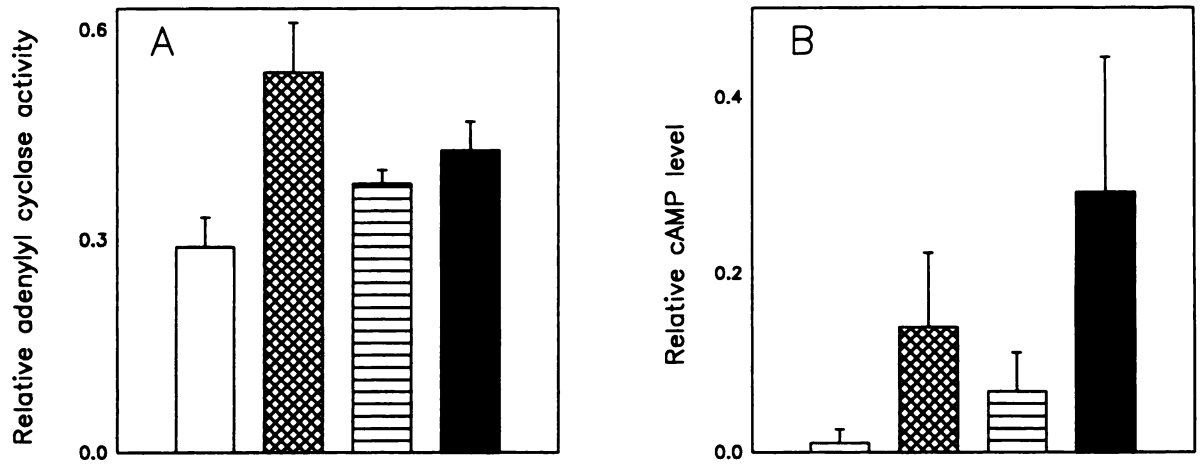

Fig. 10. Effect of βAR ligands on β_2AR activity in the presence of forskolin. Membrane cAMP production (A) and intracellular cAMP concentration (B) were measured in the presence of 1 μM forskolin in Sf9 cells infected for 48 hr with either wild-type (□) or recombinant baculovirus encoding the β_2AR in the absence of βAR ligands (■), in the presence of 10 μM propranolol (■), or in the presence of 10 μM pindolol (■). Data from individual experiments were scaled as a fraction of the signal observed in the presence of 1 μM isoproterenol, as described in the legend to Fig. 8 (membranes, 70 ± 40 pmol/mg of protein/min; whole cells, 8.8 ± 2.5 pmol of cAMP/sample). The scaled data from two independent experiments in A and three independent experiments in B were averaged to yield the values indicated. *Error bars*, standard deviations. Additional details are described in Materials and Methods.

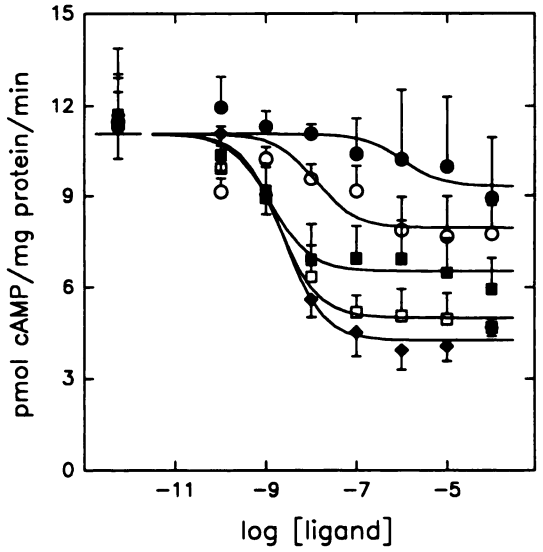

Fig. 11. Inhibition of agonist-independent adenylyl cyclase activity in membranes from CHW cells expressing β_2AR. Production of cAMP was measured in the presence of labetalol (●), pindolol (○), alprenolol (■), propranolol (□), or timolol (♦), at the concentrations indicated on the *abscissa*. The data presented represent the mean values of three experiments carried out in duplicate. The averaged data sets were analyzed simultaneously, assuming a common value of $y_{x=0}$. The fitted values of EC_{50} are as follows: labetalol, 1.1 ± 1.7 μM; pindolol, 13 ± 10 nM; alprenolol, 1.3 ± 0.7 nM; propranolol, 2.0 ± 0.8 nM; timolol, 2.5 ± 0.9 nM.

It seems likely that the interactions between βAR ligands and the β_2AR revealed using the baculovirus/Sf9 expression system accurately mimic the process of βAR transduction in mammalian systems. βAR expressed in Sf9 cells exhibit normal pharmacological properties and undergo phosphorylation and palmitoylation in the expected manner (11, 20). Furthermore, the binding affinities of antagonists obtained in the present study are consistent with those reported in membranes prepared from βAR-expressing mammalian cells (10, 21) and tissues (22–24). Inverse agonism has been demonstrated in this study in both CHW and Sf9 cells, indicating that the phenomenon can occur in both mammalian and insect cells and is not dependent upon the baculovirus/Sf9 expression system *per se*. The rank order of βAR ligands, moreover, appears to be the same with respect to both potency and inverse efficacy in both systems (Fig. 11; Table 1).

Spontaneous activity of β_2AR in Sf9 cells. The results shown in Figs. 1 and 2 indicate that the β_2AR activates adenylyl cyclase in an agonist-independent manner in membranes from Sf9 cells. In accord with the present results, purified βAR was shown to stimulate the GTPase activity of G_s in a reconstituted system, even in the absence of agonist (25). Also, the effect of the β_2AR on intracellular cAMP in Sf9 cells (Fig. 8) agrees with the results of a previous study (10) in showing that wild-type β_2AR can exhibit spontaneous activity in intact systems. These findings indicate that βAR can assume an activated state even when the ligand binding site is vacant.

Spontaneous activity also has been observed in membranes from cells expressing δ-opioid receptors, as implied by the inhibitory effects of some antagonists on basal GTPase activity (4). In contrast, the same antagonists were found to be without effect on the production of cAMP in intact cells in the absence of agonist (5). It has been suggested that the reversal of spontaneous receptor activity in an intact system by antagonists would support the idea that the biological effects of such ligands reflect their negative intrinsic activity (2). The present results show that spontaneous activity of an unliganded G protein-coupled receptor can occur in whole cells and that this activity can be inhibited by antagonists (Fig. 8). This suggests that the physiological effects of some antagonists can be ascribed at least partially to their negative intrinsic activity.

Negative intrinsic activity of βAR ligands. The results of the present study reveal that different βAR antagonists inhibit to varying degrees the spontaneous activity of the β_2AR in membranes (Fig. 6; Table 1). These ligands generally are not reported to inhibit basal adenylyl cyclase activity in such preparations (e.g., Refs. 26–28). However, in accord with the present results, propranolol, timolol, and pindolol were found to inhibit guanosine-5′-(β,γ-imido)triphosphate-stimulated adenylyl cyclase in membranes from turkey erythrocytes (7). In contrast to the present results, dichloroisoproterenol has been found in some cases to stimulate cAMP production in membranes (28, 29).

Antagonists that yielded the highest values of E_{inv} in membranes (i.e., timolol and propranolol) decreased intracellular cAMP, whereas those exhibiting the lowest values of E_{inv} in membranes (i.e., dichloroisoproterenol and labetalol) were clearly stimulatory in whole cells (Fig. 8). βAR antagonists generally are found to be without effect on intracellular cAMP (10, 21, 30–32), although dichloroisoproterenol, pindolol, and alprenolol have been reported to stimulate adenylyl cyclase activity in intact cells in some studies (10, 21). In contrast to the present results, previous studies seemingly have failed to detect the inhibition of spontaneous β_2AR activity in whole cells.

In both membranes and whole cells, the inconsistent observations with βAR antagonists may reflect the difficulty of measuring the effects of these ligands on spontaneous receptor activity. The feasibility of quantifying such effects constitutes one advantage of the baculovirus system, owing to the relatively high spontaneous activity associated with high levels of β_2AR expression. If "neutral" results are omitted, the available data, including the present results, may be summarized as follows: 1) propranolol and timolol inhibit adenylyl cyclase both in membranes and in whole cells, 2) alprenolol, labetalol, and pindolol inhibit activity in membranes and stimulate activity in whole cells, and 3) dichloroisoproterenol is inhibitory or stimulatory in membranes and stimulatory in whole cells.

In membranes from Sf9 cells, the net stimulation of adenylyl cyclase by agonists and the net inhibition of that activity by inverse agonists both are dependent upon the density of β_2AR (Figs. 2 and 5, respectively). The present results suggest that more receptors may be required for the detection of inhibition than are needed to observe stimulation, because a slight stimulatory response to isoproterenol usually could be detected 12 hr after infection of cells with the recombinant baculovirus (approximately 30 fmol of β_2AR/mg of membrane protein), whereas inverse agonism was first evident after a 24-hr infection (1–2 pmol of β_2AR/mg of membrane protein). This apparent discrepancy is not surprising, because inhibition is inherently more difficult to detect than stimulation. It is likely that inverse agonism occurs at low receptor levels, even though it is difficult to quantitate. Alternatively, one might interpret the apparent requirement for large numbers of receptors as evi-

dence that inverse agonism does not occur in nature, because endogenous levels of G protein-coupled receptors would be below the required threshold. Several lines of evidence, however, argue against that notion. 1) Inverse agonism has been observed in preliminary experiments in this laboratory with two other mammalian cell lines, expressing approximately 200 and 800 fmol of β_2AR/mg of protein.[1] 2) Endogenous receptors appear to be regulated directly by antagonists, because inverse agonism in membrane preparations has been observed with βAR in avian erythrocytes (7) and muscarinic receptors in porcine (9) and hamster hearts.[2] 3) The specific binding of ^{125}I-lysergic acid diethylamide to serotonergic sites in rat choroid plexus homogenates reveals a receptor density of 3.1 pmol/mg of protein (33), which is consistent with the density of β_2AR associated with inverse agonism in the present study. Moreover, although G protein-coupled receptors typically are found to occur at femtomole/milligram of protein levels in homogenates of mammalian tissue, the actual densities in individual neurons, or indeed at synaptic clefts, are uncertain. 4) Levitzki (34) has calculated that agonist occupancy of <0.02% of βAR in turkey erythrocyte or rat parotid gland is sufficient to saturate the relevant protein kinase and protein kinase-dependent processes that lead to agonist-induced changes in cellular activity; it follows that a level of spontaneous receptor activity too low to quantitate reliably may still have a substantial impact on cellular function. Taken together, the foregoing points attest to the potential importance of the inhibition of spontaneous receptor activity by inverse agonists in the pharmacological regulation of G protein-coupled receptors *in vivo*.

Several of the βAR ligands used in the present study are reported to act as partial agonists *in vivo*. Alprenolol, dichloroisoproterenol, labetalol, and pindolol all exhibit ISA (21, 35), a phenomenon wherein βAR blockade is observed at high rates of sympathetic activity but stimulatory effects, such as increased heart rate, may be observed at rest (21, 36). Timolol and propranolol, which lack ISA, are inhibitory in both membranes and whole cells. In contrast, alprenolol, pindolol, labetalol, and dichloroisoproterenol, ligands with ISA, tend to be inhibitory in membranes (7) (Figs. 3–7 in this study) and stimulatory in whole cells (10, 21) (Fig. 8 in this study). In light of such observations, it is unclear whether these drugs are agonists or antagonists; moreover, the difference between an agonist and an antagonist no longer seems obvious. It has been suggested that the efficacy of a ligand at a particular receptor can vary depending upon which G protein is coupled to the receptor (5), and this may account for some of the observed differences between cell types. However, it is unlikely that it could explain the differences between membranes and whole cells in the present study, because the same cells were used for both types of experiment.

The correlation observed between values of E_{inv} in membranes and whole cells (Fig. 9) may be of some help in resolving the present paradox. Although it is not understood how one ligand is able to stimulate the activity of a receptor under one set of experimental conditions and inhibit the same receptor under a different set of conditions, the correlation suggests that similar mechanisms may underlie both processes. The increase in intracellular cAMP in Sf9 cells occurred only with ligands exhibiting ISA, whereas those lacking ISA were inhibitory. In addition, E_{inv} in membranes is inversely correlated with ISA ($r^2 = -0.94$).[3] In practical terms, the effects of a novel βAR ligand on adenylyl cyclase activity in β_2AR-expressing Sf9 cells and in membranes derived from those cells may be of value in predicting its level of ISA *in vivo*. There was no obvious relationship between E_{inv} and either the lipophilicity or the local anesthetic ("membrane-stabilizing") properties of the ligands tested (37).

Mechanistic implications. The two-state scheme shown below depicts a system wherein a receptor spontaneously assumes either an inactive state (R) or an active state (R*), with the equilibrium between these being described by the unimolecular constant K_S ([R*]/[R]).

$$\begin{array}{ccc} & \overset{K_L}{} & \\ L + R & \rightleftharpoons & LR \\ \Updownarrow K_S & & \Updownarrow \alpha K_S \\ & \underset{\alpha K_L}{} & \\ L + R^* & \rightleftharpoons & LR^* \end{array}$$

A given ligand (L) binds to R and R*, to yield the association constants K_L (i.e., [LR]/[L][R]) and αK_L (i.e., [LR*]/[L][R*]), respectively; L is assumed to have no other effect on either R or R*. If α is >1, then L binds with greater affinity to the active form of the receptor and increases the number of receptors in the active state (i.e., [R*] + [LR*]), thereby stimulating the activity of the receptor (i.e., L is an agonist). If α is <1, the number of receptors in the inactive state is increased by L (i.e., L has negative intrinsic activity). The influence of any auxiliary proteins is not directly considered. This model is analogous to that proposed for the benzodiazepine receptor (1, 3, 38) and also to the ternary scheme proposed for G protein-coupled receptors (5), at least under conditions where the ligand does not appreciably affect the concentration of free G protein.

At the root of such models is the notion that the biochemical effect of a ligand-receptor interaction reflects the relative preference of a ligand for the "active" or the "inactive" form of the receptor. The stimulatory effects of agonists thus are attributed to their preference for the active receptor, which leads to an increase in the proportion of receptors in that form. The effect of antagonists can be either negative or neutral; those that favor the inactive form of the receptor exhibit negative intrinsic activity, and those with no preference lack intrinsic activity. In the presence of an activating ligand, both types of antagonist appear inhibitory. Also, in the absence of any receptor in the active form, both appear to be without effect.

Not all ligands tested in the present study inhibit cAMP production in membranes to the same extent (Fig. 6; Table 1), and their effects in whole cells similarly exhibit a range of efficacies (Fig. 8). Taken separately, each of these observations is consistent with the notion that a ligand can modulate the function of a receptor by binding preferentially to either the active or the inactive state. Taken together, however, the present results suggest that some ligands can act as agonists (i.e., $K_L < \alpha K_L$) in whole cells and as antagonists (i.e., $K_L > \alpha K_L$) in membranes prepared from the same cells; it is difficult to reconcile this observation with the notion that a ligand pro-

[1] P. Chidiac, S. Parent, and M. Bouvier, unpublished observations.
[2] P. Chidiac and J. W. Wells, unpublished observations.
[3] The correlation coefficent was calculated using ISA values listed by Waller (35) for alprenolol, pindolol, and labetalol (dilevalol), assuming an ISA of 0.0 for both propranolol and timolol (35, 39).

duces its effect by binding preferentially to either the active or the inactive form of a receptor, particularly if the only effect of the ligand is to "stabilize" one of two states.

Because expression of the β_2AR is associated with a 4.5-fold increase in adenylyl cyclase activity in membranes but only a 2.8-fold increase in whole cells, it appears that the unliganded receptor may be more "active" in membranes than in whole cells. In terms of the model described above, K_S is greater in membranes than in whole cells. An analogous conclusion was reached with respect to the function of the δ-opioid receptor in membranes and whole cells when inverse agonism was not reliably observed in the latter (4, 5). Such a difference between membranes and whole cells could account for the apparent loss of activity of an antagonist in cells, as was observed with alprenolol in the present study. The divergent effects of labetalol and dichloroisoproterenol in membranes versus whole cells, however, may be indicative of a more complex system. In terms of the scheme shown above, a single ligand will inhibit a receptor under one set of conditions and stimulate it under another set of conditions only if the ligand binds preferentially to R^* in one case and to R in the other. Alternatively, more than two states of the β_2AR may be involved in mediating the effects of βAR ligands.

Although any configuration of the β_2AR occurring in membranes presumably could arise in whole cells, the converse is not necessarily true. The discrepancies observed between the effects of βAR ligands in whole cells and membranes thus may reflect the existence of some receptor-associated component(s) present in the former but absent from the latter. It is possible that, in addition to the soluble constituents of the cell, some membrane-associated components were lost during the preparation of the membranes used in the present study. Current studies in this laboratory are aimed at identifying any cellular constituents that may, when added to membrane adenylyl cyclase assays, lead to βAR responses that more accurately reflect signal transduction in whole cells.

Acknowledgments

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