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Negative Antagonists Promote an Inactive Conformation of the β_2-Adrenergic Receptor

PHILIPPE SAMAMA, GANG PEI, TOMMASO COSTA, SUSANNA COTECCHIA, and ROBERT J. LEFKOWITZ

Departments of Medicine (Cardiology) and Biochemistry and the Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710 (P.S., G.P., R.J.L.), the Laboratorio di Farmacologia, Istituto Superiore di Sanità, 00161 Roma, Italy (T.C.), and the Institut de Pharmacologie et Toxicologie, Faculté de Médecine, 1005 Lausanne, Switzerland (S.C.)

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SUMMARY

The β_2-adrenergic receptor undergoes isomerization between an inactive conformation (R) and an active conformation (R*). The formation of the active conformation of the receptor molecule can be promoted by adrenergic agonists or by mutations in the third cytoplasmic domain that constitutively activate the receptor. Here we show that, of several β-adrenergic receptor-blocking drugs tested, only two, ICI 118551 and betaxolol, inhibit the basal signaling activity of the β_2-adrenergic receptor, thus acting as negative antagonists. We document the molecular properties of the more efficacious ICI 118551; (i) it shows higher affinity for the inactive form of the receptor and (ii) it inhibits the spontaneous formation of a β-adrenergic receptor kinase substrate by the receptor. These properties are opposite those of adrenergic agonists, indicating that, in a fashion reciprocal to that of agonists, negative antagonists promote the formation of an inactive conformation of the receptor.

The β_2-AR, which activates adenylate cyclase via the intermediation of G_s, is prototypical of the large superfamily of G protein-coupled receptors. These receptors are characterized by a highly conserved seven-membrane-spanning domain topography (1). Like many other receptors, the β_2-AR is thought to adopt two interconvertible states, one active and the other inactive (reviewed in Ref. 2).

Recently, we described mutant forms of the α_1-, β_2-, and α_2-ARs that are constitutively active, i.e., whose expression leads to significant G protein and effector activation, even in the absence of agonists (3–6). Thus, these CAM receptors mimic the active, agonist-occupied form of the WT receptors, and they are assumed to spontaneously isomerize to an active conformation (R*) capable of activating G proteins. This isomerization reaction, revealed by the unusual properties of CAM receptors, takes place in WT receptors as well; agonists bind preferentially to and thus stabilize the active conformer of the receptor (R*), thereby promoting its interaction with the G protein, which leads to biological activity (5). Thus, this conceptualization of agonist action incorporates features of both the "allosteric" and "ternary complex" models of receptor activation (2, 7, 8).

Basal, agonist-independent signaling activity of receptors has been documented previously. Although "competitive" antagonists are thought to act by blocking the access of the agonist binding site through steric hindrance, and thus have no effect on basal activity, "negative" antagonists (i.e., "inverse agonists") have the defining property of inhibiting such agonist-independent activity. For ion channel receptors, whose activity is generally regarded as being governed by allosteric transitions between an inactive (closed) state and an active (open) state, negative antagonists are thought to promote the inactive conformation at the expense of the active one (9). For G protein-coupled receptors, negative antagonists have been shown to provoke the dissociation of spontaneously occuring R-G complexes (10–12). However, in view of our current understanding of receptor activation by agonists, the mode of action of negative antagonists requires a reassessment. In the present work, we take advantage of the existence of a CAM β_2-AR to identify two β-AR ligands as negative antagonists. Their properties are then interpreted in the framework of the allosteric-ternary complex model.

Experimental Procedures

Materials. The following materials were purchased: ITS (Collaborative Research Inc.), F12 medium (GIBCO), (−)-isoproterenol and alprenolol (Sigma), ^{125}I-CYP (New England Nuclear), (−)-propranolol and PIN (Research Biochemicals Inc.), and ICI 118551 (Cambridge Research Biochemicals). The following compounds were gifts from the

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ABBREVIATIONS: AR, adrenergic receptor; WT, wild-type; CAM, constitutively active mutant; βARK, β-adrenergic receptor kinase; CYP, cyanopindolol; PIN, pindolol; Gpp(NH)p, guanylylimidodiphosphate; SDS, sodium dodecyl sulfate; CHO, Chinese hamster ovary; ITS, insulin-transferrin-selenous acid.

indicated companies: betaxolol (Synthelabo), butoxamine (Burroughs Wellcome), practolol (Ayerst), dichloroisoproterenol (Lilly), isoxsuprine (Mead Johnson), and oxprenolol (Ciba).

Adenylyl cyclase assays. The establishment of CHO cell clones expressing either WT or CAM β_2-ARs was described previously (5). Cells were cultivated in F12 medium supplemented with ITS, a serum-replacement additive. Cell membranes were prepared, and adenylyl cyclase was assayed as described (5). Briefly, freshly prepared membranes (10–30 μg of membrane protein) were incubated in 50 μl of assay mixture containing 20 mM Tris·HCl, pH 7.4, 0.8 mM EDTA, 4 mM $MgCl_2$, 0.12 mM ATP, 0.05 mM GTP, 0.1 mM cAMP, 2.7 mM phosphoenolpyruvate, 0.05 IU/ml myokinase, 0.01 IU/ml pyruvate kinase, [α-^{32}P]ATP (~3 × 10^6 dpm/tube), and other reagents as indicated in the text. Because basal activities were measured in many of the studies, incubations were extended up to 2.5 hr to increase the signals. Reactions were carried out at 37° and terminated by the addition of 1 ml of an ice-cold solution containing 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (~20,000 dpm). [α-^{32}P]cAMP was isolated as described previously (5). All points were determined in triplicate.

Ligand binding. Binding was performed and analyzed as described (5). Briefly, ^{125}I-CYP binding was assayed in 20 mM Tris·HCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM ascorbic acid, at a concentration of ~80 pM. The receptor concentration was ~8 pM. All determinations were in duplicate. Binding isotherms were analyzed by nonlinear least-square regression, according to the law of mass action (13). Affinity values were compared using a two-tailed t test.

Purification and reconstitution of mutant β_2-ARs. The cDNAs encoding the WT and CAM β_2-ARs (5, 14) were subcloned into vector pAcC4 and co-transfected in Sf9 cells with linearized viral DNA (Baculogold transfection kit; Pharmigen), to produce recombinant baculovirus. Positive viral clones were isolated by plaque assay, and receptor expression was measured by ^{125}I-CYP binding. WT and CAM β_2-ARs were prepared from Sf9 cells as described previously (15). Briefly, receptors were solubilized in 20 mM Tris·HCl, pH 7.5, 1.5% digitonin, 100 mM NaCl, 2 mM EDTA, purified on an alprenolol-Sepharose column, reconstituted in phosphatidylcholine vesicles as described (10), and stored at −70°.

Receptor phosphorylation. Recombinant βARK1 was purified from baculovirus-infected Sf9 cells as described (16). The reconstituted receptors were phosphorylated by βARK1 as described previously (17). Briefly, purified receptor preparations were incubated at 30° for 20 min with βARK1 (30 nM) in 20 mM Tris·HCl, pH 8.0, 2 mM EDTA, 10 mM $MgCl_2$, 1 mM dithiotreitol, 100 μM [γ-^{32}P]ATP (~2000 cpm/pmol), in the absence or presence of the indicated drugs. At appropriate time intervals, reactions were stopped with an equal volume of 2× SDS sample-loading buffer (8% SDS, 25 mM Tris·HCl, pH 6.5, 10% glycerol, 1% mercaptoethanol, and 0.005% bromophenol blue) and were electrophoresed on 10% SDS-polyacrylamide gels. Phosphorylation stoichiometries were determined either by excising and counting the receptor bands or by using a PhosphoImager (Molecular Dynamics).

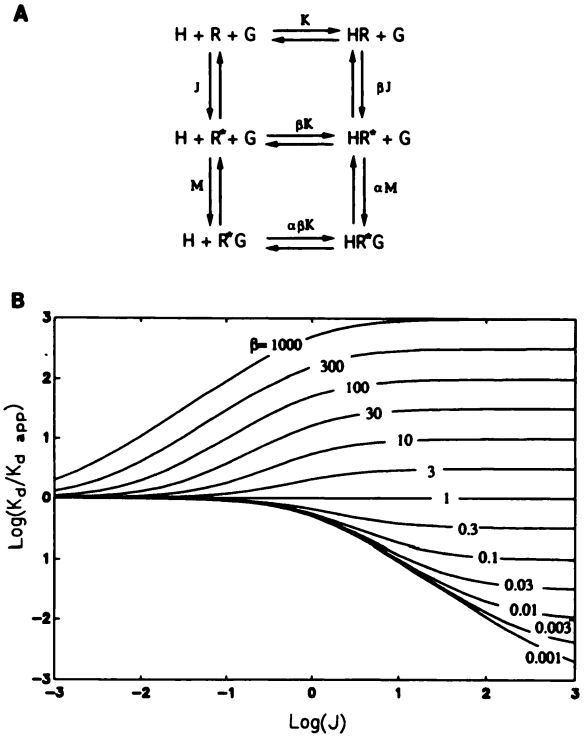

Fig. 1. Drug action in the allosteric-ternary complex model of receptor activation. A, The model was described in detail previously (5). *H*, hormone; *R* and *R**, inactive and active conformations of the receptor, respectively; *G*, G protein; *K* and *M*, bimolecular affinity constants; β and α, dimensionless constants gauging the effects of hormone binding on receptor isomerization and receptor-G protein coupling, respectively. B, Simulated effect of alterations of the *J* parameter on the apparent affinity of drugs of varying efficacies for a receptor in the absence of G protein. Based on the allosteric-ternary complex model and according to methods described in detail previously (5), the ratio of the microscopic binding affinity K_d (K_d = 1/*K*, with *K* defined as in A) to the apparent binding affinity K_{dapp} was calculated for continuously varying values of *J* (the isomerization constant) and discrete values of β (the efficacy of drugs) when [G] = 0, according to the equation $K_d/K_{dapp} = (1 + \beta J)/(1 + J)$. Each *line* was calculated for a different value of β. When *J* increases by 2 orders of magnitude (e.g., from 0.1 to 10), thus simulating a comparison between the WT (low *J*) and CAM (high *J*) β_2-ARs, contrasted patterns of binding are generated; the apparent affinity of agonists ($\beta > 1$) is greater than their true affinity, and greater than the apparent affinity for a WT (low *J*) receptor, in proportion to the value of β. The binding of neutral competitive antagonists ($\beta = 1$) is unaffected by increases of *J*. For negative antagonists ($0 < \beta < 1$), the apparent affinity for a receptor with a high *J* value is less than the true affinity and less than the apparent affinity for a WT (low *J*) receptor.

Results

Negative antagonists predicted by the allosteric-ternary complex model of receptor activation. We have recently proposed an extension of the ternary complex model to account for the existence and properties of CAM ARs (5). Briefly, this model assumes that the receptor undergoes an isomerization between an inactive conformation (R) and an active (R*) conformation, an intrinsic reaction gauged by a *J* parameter (Fig. 1A). One aspect of agonist efficacy at ARs is to drive the receptor molecule into the active conformation (gauged by a β parameter); the other aspect is to drive the binding of the hormone-receptor complex to the G protein (gauged by an α parameter) (Fig. 1A). This framework predicts the existence of a class of ligands whose binding to the receptor has a negative heterotropic effect on R/G interaction and/or receptor isomerization (i.e., α and/or β are less than 1). Because there is a spectrum of negative efficacies (i.e., a range of values for α and β), several antagonists with negative efficacies might be found, differing in the extent of maximal inhibition of adenylyl cyclase. Moreover, binding properties of a negative antagonist should be opposite those of an agonist. We simulated the binding of various drugs to a receptor able to isomerize between an active and an inactive state, in the absence of G protein (Fig. 1B). It can be seen that an increase of the *J* parameter results in changes of affinity for drugs that are related to their efficacy. For agonists ($\beta > 1$) (Fig. 1B, *upper curves*) an increase in *J* translates into an increase in affinity, for neutral antagonists ($\beta = 1$) the affinity is unchanged, and for negative antagonists ($\beta < 1$) (Fig. 1B, *lower curves*) the

effect is reversed, in that a receptor with a higher J parameter is expected to have a lower affinity for these agents. Therefore, because the primary effect of the constitutively activating mutation of the β_2-AR is to favor the isomerization of the receptor to the active state, described by an increase of the J parameter (5), a negative antagonist should display less apparent affinity for the CAM receptor than for the WT receptor.

Inhibition by two drugs of the basal signaling activity of the β_2-AR. We took advantage of the high agonist-independent adenylyl cyclase activity stimulated by the mutant β_2-AR in CHO cell membranes to screen antagonists for negative activity. Although some β-blocking agents were found to have weak agonist activity in this system (e.g., alprenolol, practolol, dichloroisoproterenol, isoxsuprine, oxprenolol, and PIN) (data not shown), two compounds lowered the basal adenylyl cyclase activity in the membranes of CHO cells harboring the mutant β_2-AR, i.e., ICI 118551, a potent β_2-selective antagonist, and betaxolol, a β_1-selective antagonist (Fig. 2A). ICI 118551 was more efficacious in inhibiting the basal activity. The activity of drugs (positive or negative) in cell membranes harboring the CAM β_2-AR was consistent with that found in cell membranes harboring the WT β_2-AR, but with a greater magnitude (data not shown). Propranolol had no consistently measurable partial agonist or negative antagonist activity at either WT or mutant receptors and thus behaved like a neutral antagonist.

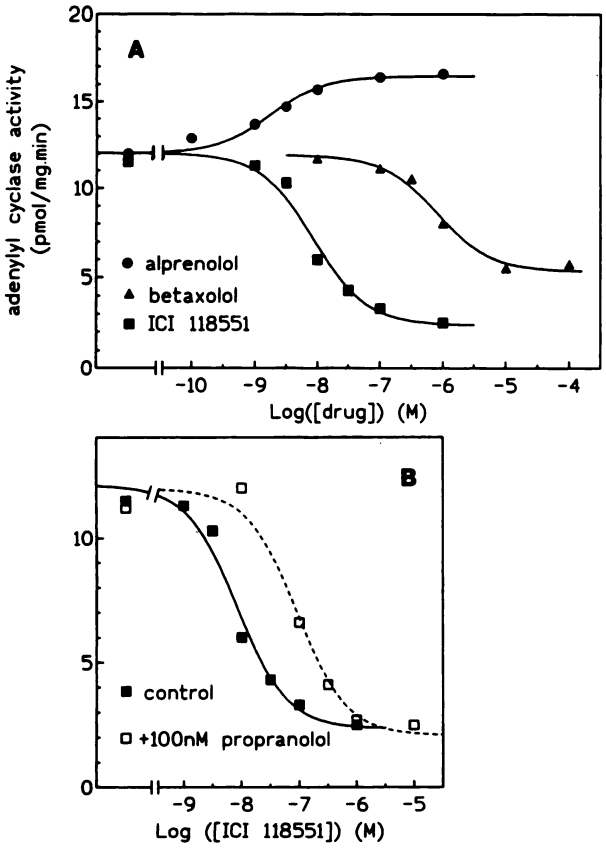

Fig. 2. Effect of various β-AR ligands on adenylyl cyclase activity. A, The effect of various AR-blocking agents on the activity of adenylyl cyclase was examined in cell membranes harboring the CAM β_2-AR at 100 fmol/mg of membrane protein. EC_{50} values were 2.5 nM, 794 nM, and 7.9 nM for alprenolol, betaxolol, and ICI 118551, respectively. In the experiment shown, the maximal activity induced by the full agonist isoproterenol (10 μM) was 65 pmol/min × mg of membrane protein. Data shown are representative of four or five such experiments, done in triplicate. B, Competition of ICI 118551 with propranolol. The inhibition of the basal activity of adenylyl cyclase by ICI 118551 was assayed in the absence (EC_{50} = 8 nM) or presence of 100 nM propranolol (EC_{50} = 100 nM). Data shown are representative of three similar experiments, done in triplicate.

To investigate the properties and mode of action of negative antagonists, we focused our work on the more efficacious ICI 118551. The inhibition of basal activity by ICI 118551 was competitively inhibited by propranolol, indicating that the inhibitory effect of this compound is mediated by the receptor (Fig. 2B).

Binding properties. We then examined the ligand-binding properties of ICI 118551 by competition with the radioligand ^{125}I-CYP. ICI 118551 had significantly ($p < 0.05$) lower affinity for the CAM β_2-AR (K_i = 1.3 ± 0.3 nM, four experiments) than for the WT receptor (K_i = 0.59 ± 0.04 nM, three experiments) (Fig. 3). This behavior is in sharp contrast to that of agonists, which exhibit a higher affinity for the CAM β_2-AR, in proportion to their intrinsic activity (5). It is consistent with the idea that ICI 118551 drives the receptor into its inactive conformation, thus acting in a fashion that is reciprocal to that of agonists. Guanine nucleotides had no appreciable effect on experimental binding isotherms (data not shown). Therefore, the binding properties of ICI 118551 do not reflect the interaction of the mutant receptor with the G protein. Likewise, no binding heterogeneity could be detected. This is reminiscent of our previous observation that partial agonists as efficacious as ephedrine (with an intrinsic activity of ~0.5), in the same expression system, had similarly monophasic, Gpp(NH)p-insensitive binding isotherms. Only with stronger agonists (e.g., dobutamine, salbutamol, and isoproterenol) were biphasic, Gpp(NH)p-sensitive binding isotherms observed (5). The present case with ICI 118551, as with partial agonists, probably reflects a technical limitation where computer curve-fitting cannot distinguish between one- and two-site fits. This is consistent with the notion that ICI 118551 might be a partial negative antagonist (see below).

Receptor phosphorylation. We next tested the ability of the purified and reconstituted WT and CAM β_2-ARs to serve

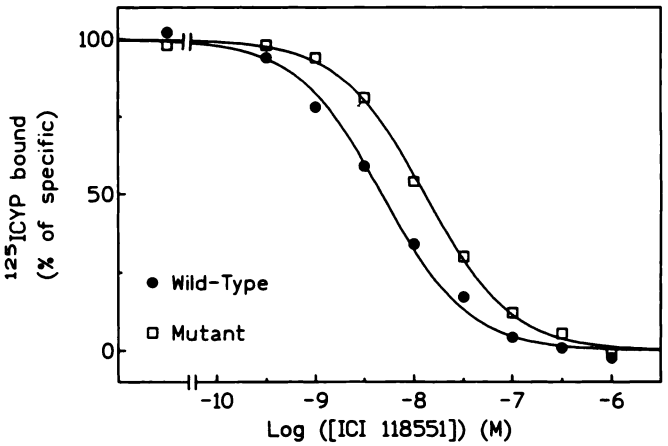

Fig. 3. Binding of ICI 118551 to the WT and CAM β_2-ARs. Competition binding experiments were performed with ICI 118551 and the radioligand ^{125}I-CYP, as described in Experimental Procedures, on CHO cell membranes harboring either the WT or the mutated form of the β_2-AR at 100 fmol/mg of membrane protein. The K_i for the WT receptor was 0.60 nM; the K_i for the mutant receptor was 1.5 nM. Total and nonspecific binding were ~8000 cpm and ~1000 cpm, respectively. Shown is a representative of three similar experiments, done in duplicate.

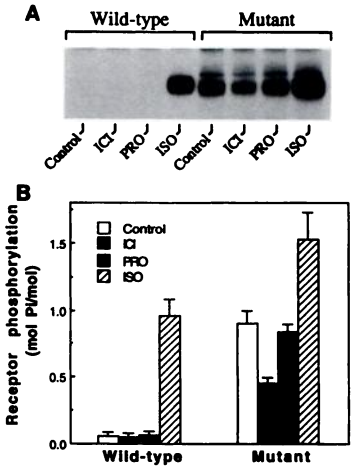

Fig. 4. Effect of AR ligands on βARK-mediated phosphorylation of the β_2-AR. A, WT or mutant β_2-ARs were reconstituted in phospholipid vesicles, phosphorylated by βARK1 in the presence or absence of the indicated agents, and electrophoresed on SDS-polyacrylamide gels (see Experimental Procedures). *ISO*, (−)-isoproterenol; *PRO*, (−)-propranolol; *ICI*, ICI 118551. All drugs were used at a concentration of 100 μM. Shown is a representative of three such experiments. B, Stoichiometries of P_i incorporation. Shown are the means ± standard errors of three experiments done in triplicate.

as substrates for recombinant βARK1 (Fig. 4). As documented in the past, unoccupied WT receptors showed virtually no detectable phosphorylation, whereas agonist-occupied receptors were phosphorylated (18). This situation presumably reflects the fact that very little spontaneous isomerization of the WT receptor to the active conformation occurs. In striking contrast, however, considerable agonist-independent phosphorylation of the CAM β_2-AR was noted (Fig. 4). Additional phosphorylation occurred upon isoproterenol addition. These results, obtained *in vitro* with a mutant β_2-AR, are in agreement with our previous observation that CAM α_2-ARs exhibit elevated agonist-independent, βARK1-mediated phosphorylation in a whole-cell assay (6). Morever, whereas the antagonist propranolol did not affect the level of agonist-independent phosphorylation of the CAM β_2-AR, the negative antagonist ICI 118551 clearly decreased it. This presumably reflects the ability of ICI 118551 to inhibit the accumulation of the active conformer of the receptor. The extent to which an agonist induces the formation of a βARK substrate by the β_2-AR was previously shown to correlate remarkably with its ability to stimulate adenylyl cyclase (18). Assuming the same correlation for a negative antagonist, the fact that ICI 118551 inhibits only ~50% of the βARK1-mediated phosphorylation of the CAM β_2-AR suggests that this compound has less than maximal (negative) efficacy.

Discussion

The defining property of negative antagonists is their ability to inhibit the spontaneous activity of receptors. *In vivo*, however, this hormone-independent activity is probably masked by the activity stimulated by low levels of circulating epinephrine (19, 20). Thus, the blockade of a tonic hormonal activation of the receptor by a purely neutral antagonist might mimic negative antagonism (21). Identifying negative antagonists therefore requires careful control of the experimental system, such as is provided in the present work by *in vitro* cell cultures in the absence of serum.

Negative activity of drugs acting at G protein-coupled receptors has been described previously and was recently reviewed (22). Its existence at β-ARs has been inconsistently documented. For instance, propranolol and PIN were shown to be negative antagonists for the β-AR-coupled adenylyl cyclase of turkey erythrocytes (23), whereas the former showed no consistent activity and the latter was a weak agonist in our system. The pharmacology of the turkey erythrocyte receptor, however, is somewhat different from that of the mammalian β_2 subtype. More intriguingly, alprenolol was shown to inhibit the basal GTPase activity of β_2-AR-coupled G_s in reconstituted phospholipid vesicles; both receptor and G protein were of mammalian origin (10). In contrast, this compound exhibited weak agonist activity at the level of adenylyl cyclase stimulation in our system. It is likely that differences in pharmacological subtype and the wide diversity of biological sources of β-AR systems used in these studies are responsible for such variances in the findings.

The molecular mode of action of negative antagonists of G protein-coupled receptors has been interpreted in light of the understanding of receptor activation. Following the concept that receptors isomerize between an active and an inactive state (reviewed in Refs. 2 and 7), Karlin (24) predicted the existence of drugs that would preferentially bind the inactive conformation of a receptor and thus inhibit spontaneous isomerization to the active state (then of unknown nature). It was subsequently shown that, for G protein-coupled receptors, the active state can be identified with a transient R-G complex, whose formation is promoted by the binding of agonists (7). The spontaneous (agonist-independent) occurrence of such R-G complexes in membranes was also quickly recognized (7), as was the existence of negative antagonists that inhibit their formation (11, 12, 25). However, phenomena reflecting drug efficacy, but independent from receptor interaction with the G protein, indicate that agonists by themselves also promote an active state of the receptor molecule. Such phenomena include (i) *in vitro* βARK-mediated phosphorylation of the receptor (18) and (ii) increased ligand binding affinity of a mutated β_2-AR for agonists (5). Accordingly, we have proposed that G protein activation is a consequence of the formation of an active (i.e., activating) conformer of the receptor (5, 8). In the present report, the same experimental approach reveals properties of a negative antagonist, ICI 118551, that are reciprocal to those of agonists. Indeed, (i) ICI 118551 has higher affinity for the inactive form of the receptor and (ii) it inhibits the spontaneous formation of the βARK substrate. The latter finding indicates that ICI 118551 inhibits receptor isomerization to the active state (i.e., $\beta < 1$; see Fig. 1A). Because such an effect is sufficient to account for the properties of this antagonist observed at the level of receptor binding and adenylyl cyclase activity, it is not clear at present whether ICI 118551 also has a negative heterotropic effect on R*/G interaction (i.e., $\alpha < 1$; see Fig. 1A). Indeed, the correlation between the activation properties of drugs and their effects on isomerization (Refs. 5 and 18 and this work) suggests that the promotion or impairment by drugs of the formation of R*-G complexes, which is the key step in the activation of the effector (adenylyl cyclase in the case of the β_2-AR), may in fact be controlled largely at the level of receptor isomerization. However, the existence of

drugs whose effects on receptor isomerization and coupling to the G protein are opposed (i.e., $\alpha > 1$ and $\beta < 1$ or $\alpha < 1$ and $\beta > 1$) cannot be ruled out at present. The existence of CAM forms of receptors should thus facilitate the investigation of the molecular basis of receptor-mediated drug action.

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Send reprint requests to: Robert J. Lefkowitz, Howard Hughes Medical Institute, P.O. Box 3821, Duke University Medical Center, Durham, NC 27710.