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Identification of Allosteric Antagonists of Receptor-Guanine Nucleotide-Binding Protein Interactions

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SUMMARY

A series of compounds that inhibit the coupling of the α_2-adrenergic receptor and the β_2-adrenergic receptor to the guanine nucleotide-binding proteins (G proteins) G_i and G_s, respectively, have been identified. This inhibition of G protein coupling was detected by the ability of the compounds to reduce the affinity of these receptors for agonists without affecting antagonist affinity. Analysis of the structure-activity relationships of these compounds revealed a requirement for regularly spaced anionic substituents on amphipathic structures for this inhibition to occur. The compounds do not interact at the ligand binding site of the receptor or at the GTP binding site of the G protein. The identification of compounds that can uncouple receptors from G proteins demonstrates the potential for the discovery of small molecule inhibitors of receptor-G protein interactions that act as allosteric antagonists at this site.

α_2AR belong to a large family of hormone and neurotransmitter receptors whose actions are mediated through the activation of heterotrimeric G proteins (1, 2). Agonist binding to the α_2AR promotes its interaction with the G protein G_i, leading to the formation of a high affinity agonist-receptor-G_i complex. Antagonists bind to the receptor but do not promote this high affinity interaction. Thus, in the absence of any interaction with G_i, the affinity of the receptor for agonists but not antagonists is reduced. Hence, agents such as GTP or its nonhydrolyzable analogs Gpp(NH)p and GTPγS, which bind to the α-subunit of the G protein and uncouple the G protein from the receptor, reduce the affinity of the receptor for agonists but not for antagonists.

Several G protein-coupled receptors have been cloned and their primary sequences determined. The receptors of this class show considerable sequence homology, presumably reflecting their common mechanism of action (2, 3). All of these receptors whose sequences are known consist of seven conserved stretches of hydrophobic amino acids, presumed to form transmembrane helices, connecting eight more divergent hydrophilic loops. Biophysical and biochemical analysis of rhodopsin (4) and genetic analysis of the βAR (5–9) have implicated residues within the conserved hydrophobic domains of these receptor proteins in their interactions with ligands. In contrast, several lines of evidence suggest the involvement of residues within the putative third intracellular loop of the receptors in the interactions with G proteins (10–14). Mutagenesis of the βAR has demonstrated that residues at the N- and C-terminal ends of the third intracellular loop of the protein are required for G protein coupling to occur (10, 11). Analysis of chimeric M_1/M_2-muscarinic acetylcholine receptors also suggests that the third intracellular loops of these receptors are involved in their coupling to G proteins (14). The analogous hydrophilic loop of rhodopsin has been implicated by biochemical and immunological studies in its interaction with the G protein transducin (4). However, examination of the primary sequences of the third intracellular loops of these receptors has not revealed an obvious G protein-binding consensus sequence. This third intracellular loop domain is one of the most structurally divergent regions among G protein-coupled receptors, varying widely in length and amino acid composition from one receptor to another (3). Furthermore, there is no striking primary sequence homology within this domain, even among receptors that activate the same G protein. We have previously noted that the regions of the βAR shown to be important for G protein coupling, the N- and C-terminal segments of the third intracellular loop, would be predicted by Chou and Fasman hydro-

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ABBREVIATIONS: α_2AR, α_2-adrenergic receptors; G protein, guanine nucleotide-binding regulatory protein; Gpp(NH)p, guanosine-5′-(β, γ-imido)triphosphate; GTPγS, guanosine-5′-*O*-(γ-thio)triphosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*, *N*, *N′*, *N′*,-tetraacetic acid; βAR, β-adrenergic receptor; CHO, Chinese hamster ovary; ^{125}I-CYP, ^{125}I-cyanopindolol.

pathicity analysis to form amphipathic α-helical structures (11). The predicted amphipathic α-helical nature of these domains would be conserved among all of the G protein-coupled receptors whose sequences are known, even though the primary sequences of these regions are divergent. Thus, we have suggested that the secondary structure of this region, specifically the putative amphipathic α-helical motif, might contribute to receptor-G protein recognition.

Recently, heparin, a polysulfonated glycosamino glycan, was reported to uncouple the α_2AR from G_i in membrane preparations from human platelets (15). Suramin, another polyanionic compound, was also reported to inhibit opioid receptor coupling to G_i (16). These observations were intriguing because one might predict that polyanions with regular spacing, such as heparin and suramin, could interact with the largely cationic charged face of the putative amphipathic helix of the α_2AR proposed to be involved in coupling to G_i. Therefore, we have analyzed the ability of a series of sulfonated glycan and aromatic compounds to inhibit receptor-G protein interactions. Several compounds of this class were identified that prevent the coupling of receptors to G proteins.

Materials and Methods

Receptor Cloning and Expression

The gene encoding the α_2AR was cloned from a human platelet cDNA library by hybridization with oligonucleotide probes based on the published DNA sequence of the gene (17). The protein was expressed in CHO cells using the SV40-based expression vector previously described for the βAR (5). The hamster βAR gene was cloned and expressed in murine L cells and CHO cells, as previously described (5). Cells were grown in monolayer culture in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, in a water-saturated 5% CO_2 atmosphere, at 37°.

Membrane Preparation

Monolayer cultures of cells were washed with cold phosphate-buffered saline, lysed in 1 mM Tris, pH 7.4, at 4° for 15 min, scraped, and pelleted at 30,000 × *g*. Membranes from cells containing the α_2AR were resuspended in 50 mM Tris, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.4 (Buffer A). Membranes from cells expressing the β_2AR were resuspended in 75 mM Tris, 12.5 mM $MgCl_2$, 1.5 mM EDTA, pH 7.5 (Buffer B). All membranes were quick-frozen in liquid N_2 and stored at −80° until use.

Ligand Binding Assays

α_2AR. Two hundred to three hundred micrograms of membrane protein were incubated in 1 ml of Buffer A with 1.2 nM concentrations of the agonist [^{3}H]UK14304 or 1.3 nM concentrations of the antagonist [^{3}H]yohimbine, at 23° for 1 hr. The binding was stopped by filtration through GF/C Whatman filters presoaked with 0.3% polyethylenimine, followed by washing with three 5-ml portions of 50 mM Tris, pH 7.4. Radioactivity was determined using Aquasol-2 in a Beckman scintillation counter. Nonspecific binding was determined in the presence of 100 μM oxymetazoline. Competition binding of nonradiolabeled agonists and antagonists was determined as described above, in the presence of 1.3 nM [^{3}H]yohimbine, with the addition of increasing concentrations of the competing ligands, in Buffer A for 60 min at 23°. The data were analyzed using the interative program LIGAND (18).

β_2AR. Binding of the antagonist ^{125}I-CYP was determined by incubating 20 μg of membrane protein in 250 μl of Buffer B containing 10–400 pM ^{125}I-CYP, for 60 min at 23°. Bound radioactivity was separated in GF/C membrane filters, which were washed with three 5-ml portions of 10 mM Tris, pH 7.5, 0.1 M NaCl, and counted in a Beckman γ-counter. Nonspecific binding was determined in the presence of 10 μM alprenolol. Competition binding of agonists and antagonists was measured in the presence of 35 pM ^{125}I-CYP and increasing concentrations of competing ligands in Buffer B, for 60 min at 23°. Data were analyzed using the LIGAND program (18).

Adenylyl cyclase assays were performed on membrane preparations by the method of Salomon *et al.* (19).

Determination of Ligand Dissociation Rates

Membranes from cells expressing the α_2AR (200–300 μg of protein) were equilibrated with 1.2 nM [^{3}H]UK14304 or 1.3 nM [^{3}H]yohimbine for 60 min at 23°. Dissociation was initiated by adding 10 μM oxymetazoline in the absence or presence of either 100 μM Gpp(NH)p, 150 μM L-451,167, or 100 μM Gpp(NH)p + 150 μM L-451,167. At various time points, bound radioligand was separated from free by rapid filtration and washing on GF/C filters, as described above. The time required for filtration and washing was approximately 20 sec.

GTPγS Binding

Phospholipid vesicles were prepared by sonication of dioleoylphosphatidylcholine and dioleoylphosphotidylserine (1:1 ratio) at a final concentration of 0.13% in Buffer C (20 mM Tris, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 100 mM NaCl), containing 0.8% sodium cholate, at 4 ° under N_2 for 10 min. Four hundred microliters of the lipid mixture were mixed with 10 μg of purified G_i/G_o (gift from Dr. M. Graziano, Merck, Sharp and Dohme Research Laboratories) and diluted gradually to 8 ml with cold Buffer C. Two milliliters of 50% polyethylene glycol-8000 was added and the suspension was mixed thoroughly and incubated on ice for 1 hr before vesicles were pelleted at 250,000 × *g* for 1.5 hr. The pellet was washed once and resuspended in Buffer C. [^{35}S]GTPγS binding to the reconstituted G protein was measured as described by Northup *et al.* (20). Briefly, reconstituted G protein containing approximately 3 pmol of GTPγS binding activity was incubated in a final volume of 100 μl of Buffer C in the presence of 50 μM mastoparan, with or without 50 μM L-451,167, for 15 min at 23 °. Binding was initiated by the addition of [^{35}S]GTPγS (2.55 Ci/mol; New England Nuclear) at a final concentration of 1 μM. The binding reaction was stopped by dilution with 2 ml of cold Buffer D (20 mM Tris, pH 8.0, 25 mM $MgCl_2$, 100 mM NaCl, 100 μM GTP) and filtration through a presoaked nitrocellulose membrane. Membranes were washed with four 2-ml portions of cold Buffer D without GTP, dried, and counted in Aquasol-2. Nonspecific binding was determined in the absence of G protein and was subtracted from the total binding.

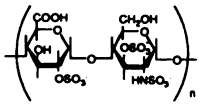
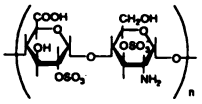
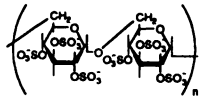
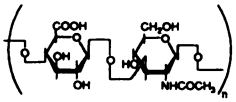
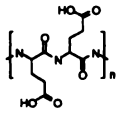
Results

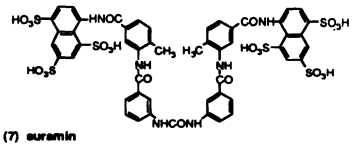
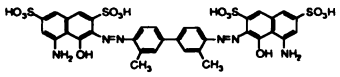
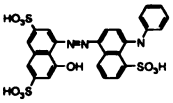
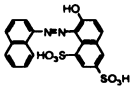
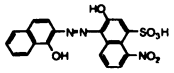
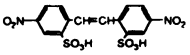
As previously reported for human platelet membrane preparations (15), heparin decreased the binding of agonists to the human α_2AR expressed in CHO cells (IC_{50} = 0.1 μM) but did not affect antagonist binding (IC_{50} > 200 μM). This decrease in agonist binding in the presence of heparin was mimicked by the related sulfonated sugar dextran sulfate but not by smaller anionic glycans, such as glucuronic acid (Table 1). Agonist binding to the α_2AR was not inhibited by the nonsulfonated polyacids hyaluronic acid or polyglutamic acid, and the inhibition was reduced when de-*N*-sulfated heparin was used (IC_{50} = 9 μM) (Table 1). Because this activity appeared to correlate with the presence of sulfonate groups on the compound, a variety of polysulfonic acids were tested for their ability to decrease the binding of agonists to the α_2AR, with the results outlined in Table 1. Suramin and trypan blue both inhibited agonist binding to the α_2AR with an IC_{50} of 2-3 μM, indicating that aromatic moieties can substitute for the glycan backbone in mediating this inhibitory activity. Compound L-451,167, a polysulfonated diazodinaphthylene dye, represents the smallest compound that inhibited agonist binding to the receptor (IC_{50}

TABLE 1

Inhibition of binding of [^{3}H]UK14304 to the α_2AR

Binding of 1.2 nM [^{3}H]UK14304 to membranes from CHO cells expressing the α_2AR was determined at 23° for 1 hr in the presence of various concentrations of the compounds listed in the table, as described in Materials and Methods. The IC_{50} was defined as the concentration of compound that inhibited [^{3}H]UK14304 binding by 50% under these conditions.

	IC_{50}
	μM
	IC_{50} (μM)
 (1) heparin	0.12
 (2) de-N-sulfated heparin	9
 (3) dextran sulfate - M, 500,000 M, 5,000	0.005 0.01
 (4) glucuronic acid	>200
 (5) hyaluronic acid	>200
 (6) polyglutamic acid	>200

	IC_{50}
	μM
 (7) suramin	2
 (8) trypan blue	3
 (9) 8-(4-anilino-5-sulfo-1-naphthylazo-1-naphthol-3, 6-disulfonic acid [L-451,167]	10
 (10) 1-hydroxy-1-methyl-2-(1-naphthyl)-ethane sulfonate	>200
 (11) 7-hydroxy-8(1-naphthylazo)-1,3-naphthalene disulfonate	>200
 (12) 3-hydroxy-4-[(1-hydroxy-2-naphthalenyl)azo]-7-nitro-1-naphthalene sulfonic acid	100
 (13) 4,4'-dinitrostilbene-2,2'-disulfonate	100

= 10 μM). Compounds having only a single naphthyl group did not affect agonist binding (cf. compound **10**, Table 1). Sulfonate substituents on both of the naphthyl groups appeared to be required for inhibition of agonist binding, as demonstrated with compounds **11** and **12.** Substitution of the naphthyl rings with phenyl moieties, decreasing the size and hydrophobicity of the sulfonated aromatic centers, also decreased the activity of the compounds (compound **13**). Thus, a brief analysis of the structural requirements for inhibition of agonist binding suggests that both the presence of the sulfonate group and the spacing of the aromatic moieties contribute to the inhibitory activities of these compounds. None of the compounds shown in Table 1 inhibited antagonist binding to the α_2AR at concentrations up to 100 μM (data not shown).

Because L-451,167 was the smallest compound tested that inhibited α_2AR agonist binding in the low micromolar range, the mechanism of inhibition by this compound was investigated further. As shown in Fig. 1A, this compound inhibited the binding of the agonist [^{3}H]UK14304 to the α_2AR with an IC_{50} of 10 μM, whereas it inhibited the binding of the antagonist [^{3}H]yohimbine only slightly at concentrations above 100 μM. The effects of this compound were not specific for the α_2AR, however. As shown in Fig. 1B, L-451,167 also inhibited the binding of the agonist isoproterenol to the β_2AR with an IC_{50} of 20 μM. In addition, the compound decreased the binding of the antagonist ^{125}I-CYP to the βAR, but with a potency >10-fold less than that with which it reduced the agonist binding ($IC_{50} > 100$ μM; Fig. 1B).

The reduction in agonist binding to the α_2AR reflects a decrease in the affinity of the agonist in the presence of L-451,167. As demonstrated by the equilibrium binding experiment shown in Fig. 2, 50 μM L-451,167 decreased the affinity of the receptor for the agonist epinephrine by 2 orders of magnitude. This decrease in agonist affinity with L-451,167 was similar to that observed in the presence of the nonhydrolyzable GTP analog Gpp(NH)p (Fig. 2). Quantitative analysis of the curves shown in Fig. 2 revealed that both L-451,167 and Gpp(NH)p increased the proportion of receptors in the low affinity binding state from the control value of 24% to approximately 70%, and the two effects were not additive (Table 2). The decreased agonist affinity was also reflected in an increase in the rate of dissociation of [^{3}H]UK14304 in the presence of L-451,167 (Fig. 3A and Table 2). In the control membranes, 62% of the agonist-receptor complex was in a slowly dissociat-

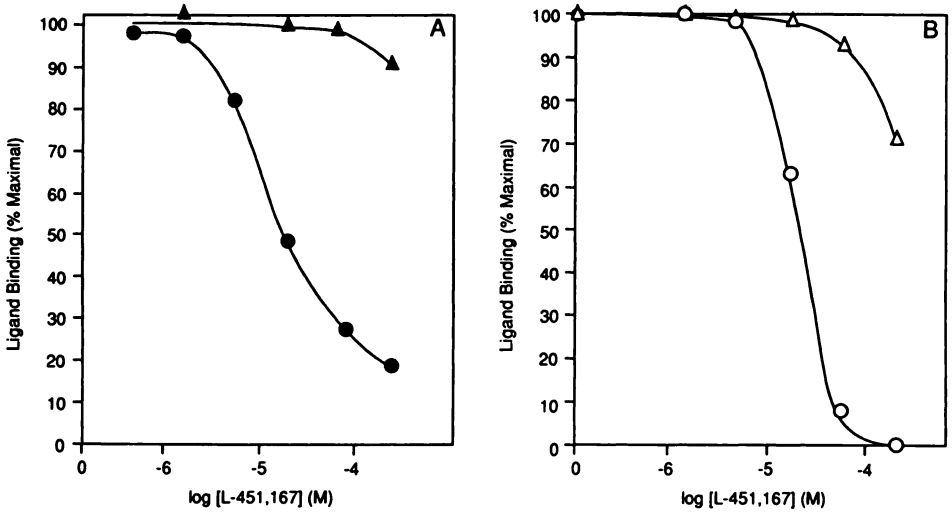


Fig. 1. Inhibition of agonist and antagonist binding to the α_2AR and β_2AR by L-451,167. A, [^{3}H]UK14304 (●) and [^{3}H] yohimbine (▲) binding to CHO cell membranes containing the α_2AR was measured in the presence of increasing concentrations of L-451,167, as described in Materials and Methods. B, Inhibition of ligand binding to membranes from L cells expressing the β_2AR by increasing concentration of L-451,167 was measured as described in Materials and Methods. Inhibition of the antagonist ^{125}I-CYP was measured directly (△), using 35 pM ^{125}I-CYP in a final volume of 250 μl. Inhibition of agonist binding (○) was determined by measuring the displacement of ^{125}I-CYP binding by various concentrations of the agonist isoproterenol. The concentrations of isoproterenol were chosen to span the IC_{50} of isoproterenol binding to the G protein-coupled form of the receptor. The isoproterenol binding presented in the figure was calculated as the displacement of 35 pM ^{125}I-CYP binding by 10^{-8} M isoproterenol, and it was verified for each point that a decrease in the displacement of ^{125}I-CYP by isoproterenol reflected a rightward shift in the agonist competition curve.

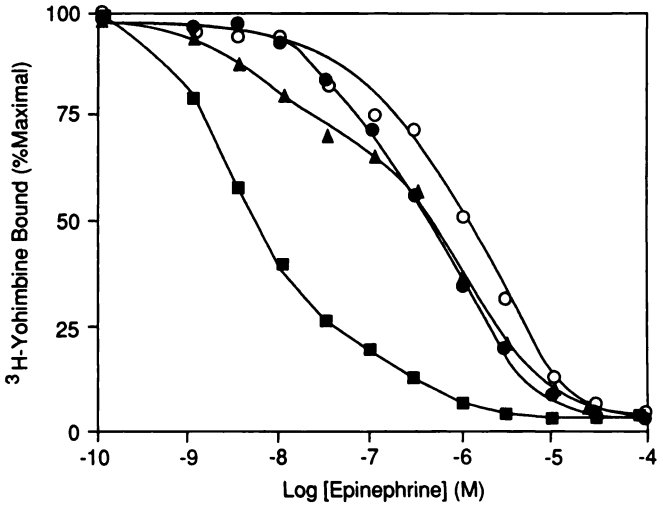


Fig. 2. Effects of L-451,167 on agonist affinity for the α_2AR. Binding of the agonist (−)-epinephrine was measured in competition with [^{3}H] yohimbine, as described in Materials and Methods, with no addition (■), 100 μM Gpp(NH)p (●), 50 μM L-451,167 (▲), or both 50 μM L-451,167 and 100 μM Gpp(NH)p (○). Data were analyzed by the LIGAND program of Munson and Rodbard (18). For the experiment shown, the receptor B_{max} values were: control, 81 pM; plus Gpp(NH)p, 81 pM; plus L-451,167, 76 pM; plus L-451,167 and Gpp(NH)p, 61 pM.

ing (high affinity) state, and 38% was in a rapidly dissociating (low affinity) state. The addition of 50 μM L-451,167 resulted in a redistribution of receptors between these states, such that 30% were in the slowly dissociating state and 70% in the rapidly dissociating form. As observed in the equilibrium binding experiment, the effect of L-451,167 on agonist dissociation from the α_2AR was similar to that of Gpp(NH)p, and the effects of the two compounds were not additive. In contrast, neither L-451,167 nor Gpp(NH)p affected the rate of dissociation of the antagonist [^{3}H]yohimbine from the receptor (Fig. 3B).

The reduction in the affinity of the receptor for agonists by L-451,167 could reflect either a direct effect of the compound on the ligand-binding domain of the receptor or a decrease in receptor-G protein interactions. To investigate the first possibility, the effects of L-451,167 on the affinity of agonists for a mutant βAR that has previously been demonstrated not to couple to G_s (11) were assessed. As shown in Fig. 4A, the wild-type βAR binds the agonist isoproterenol with two classes of affinity states, which are converted to a single class of low affinity sites in the presence of Gpp(NH)p. As observed with the α_2AR, this effect of Gpp(NH)p on the wild-type βAR was mimicked by L-451,167. As previously reported (11), D(222–229)βAR, which does not couple to G_s or activate adenylyl cyclase, binds isoproterenol with a single class of affinity sites, which are unaffected by Gpp(NH)p (Fig. 4B). Like Gpp(NH)p, L-451,167 had no effect on the affinity of this mutant βAR for agonists (Fig. 4B).

Although it does not act at the binding site of the receptor at concentrations of <50 μM, L-451,167 was not completely specific for receptor-G protein interactions. This compound also inhibited the basal, agonist-stimulated, and NaF-stimulated activation of adenylyl cyclase (data not shown), making it difficult to directly assess the functional effects of inhibition of receptor-G protein coupling in this system. The inhibition of other enzymatic processes by L-451,167 is perhaps not surprising in light of the multifactorial effects of heparin within cells.

To further define the site of inhibition by L-451,167, the effects of the compound on the GTP binding site of the G protein were determined. The rate of GTPγs binding was measured; this rate has been demonstrated to reflect the off-rate for GDP as the rate-limiting step (1). GTPγS bound to a purified preparation consisting of a mixture of the G proteins G_i and G_o, reconstituted into phospholipid vesicles, with an initial rate of 0.18 min^{-1} at 23°. Neither the initial rate nor the

TABLE 2

Affinity states of the α_2AR in the presence of L-451,167

Agonist affinity was determined either by equilibrium binding, as shown in Fig. 2, or kinetically, as in Fig. 3. Dissociation constants for the binding of epinephrine to the α_2AR in membrane preparations were determined as described in the legend to Fig. 2, using the LIGAND program (18). K_H and K_L indicate the K_d values for the high and low affinity states, and R_H and R_L the percentage of total receptors that exist in those two states, respectively. From dissociation experiments, as described in Fig. 3, k_S and k_F indicate the dissociation rates for the slowly and rapidly dissociating states of the receptor, and R_S and R_F designate the percentage of receptors in those two states, respectively, as determined by the method of Duggleby (25). The values determined for k_F represent minimum values for the rapid dissociation rate, limited by the speed of the assay. All values are the mean ± standard deviation of two or three independent experiments.

	Equilibrium			
	K_d		B_{max}	
	K_H	K_L	R_H	R_L
	M		%	
Control	$2 \pm 1 \times 10^{-9}$	$2 \pm 0 \times 10^{-7}$	76 ± 4	24 ± 4
+Gpp(NH)p	$3 \pm 2 \times 10^{-8}$	$7 \pm 1 \times 10^{-7}$	40 ± 2	61 ± 3
+L-451,167	$2 \pm 1 \times 10^{-9}$	$9 \pm 1 \times 10^{-7}$	27 ± 2	73 ± 2
+L-451,167 + Gpp(NH)p	$3 \pm 3 \times 10^{-8}$	$2 \pm 0 \times 10^{-6}$	23 ± 0	77 ± 0
	Kinetic			
	k_d		Occupancy	
	k_S	k_F	R_S	R_F
	min^{-1}		%	
Control	0.02 ± 0.01	≥0.2 ± 0.1	62 ± 14	38 ± 13
+Gpp(NH)p	0.06 ± 0.01	≥10 ± 2	33 ± 2	67 ± 3
+L-451,167	0.06 ± 0.01	≥12 ± 3	30 ± 2	70 ± 3
+L-451,167 + Gpp(NH)p	0.04 ± 0.01	≥12 ± 1	21 ± 1	79 ± 1

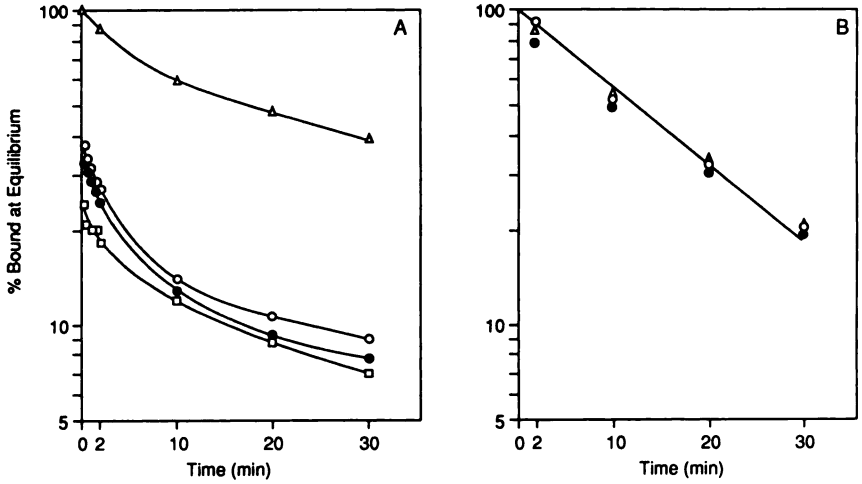


Fig. 3. Effects of L-451,167 on dissociation rates of agonists and antagonists from the α_2AR. Binding of the agonist [^{3}H]UK14304 (A) or the antagonist [^{3}H] yohimbine (B) was brought to equilibrium for 1 hr at 23°. Dissociation was initiated by the addition of 10 μM oxymetazoline and bound radioligand was measured at various time points, as described in Materials and Methods. Dissociation was determined in the absence (△) or presence of either 100 μM Gpp(NH)p (○), 150 μM L-451,167 (●), or both 150 μM L-451,167 and 100 μM Gpp(NH)p (□). The rates of dissociation of bound [^{3}H]UK14304 were analyzed by Marquardt's algorithm as described by Duggleby (25), where fast and slow phase components represent dissociation from low and high affinity binding sites, respectively.

maximal level of GTPγS binding to G_i/G_o was altered by the addition of 50 μM L-451,167. Higashijima *et al.* (21) have recently demonstrated that the wasp venom peptide mastoparan markedly stimulated the rate of GTPγS binding to G proteins without affecting the maximal level of binding, thus mimicking the effect of the activated receptor on the G protein. Therefore, we examined the effect of L-451,167 on the mastoparan-stimulated rate of GTPγS binding. As shown in Fig. 5, mastoparan stimulated the rate of GTPγS binding to G_i/G_o 3.5-fold to 0.63 min^{-1}, with no change in B_{max} observed (see the legend to Fig. 5). L-451,167 at 50 μM inhibited this mastoparan-stimulated rate by 30% (Fig. 5), while B_{max} remained unaffected. Thus, L-451,167 inhibited the mastoparan-stimulated GTPγS binding to G_i/G_o, while having no effect on the rate or level of basal GTPγS binding.

Discussion

The binding of agonists, but not antagonists, to G protein-coupled receptors causes the formation of a high affinity agonist-receptor-G protein ternary complex. In the current study, we have taken advantage of this differential association of the agonist- and antagonist-occupied forms of the α_2AR with G_i to identify compounds that interfere with receptor-G protein interactions. Such compounds would be expected to decrease the affinity of the receptor for agonists but, unlike classical inhibitors that interact with the ligand binding site of the receptor, these reagents should not affect the affinity of the receptor for antagonists. The previously reported ability of heparin to uncouple the α_2AR from G_i (15) and of suramin to decrease opioid stimulation of GTPase activity (16) suggested that polyanions might interfere with receptor-G protein coupling. A survey of other polyanionic compounds revealed that multiply substituted aromatic sulfonic acids, such as the compound L-451,167, also uncoupled the α_2AR from G_i, with the aromatic and the sulfonate moieties both contributing to the inhibitory effects of these compounds. However, this compound did not show specificity for the α_2AR, inasmuch as it also uncoupled the structurally related β_2AR from G_s.

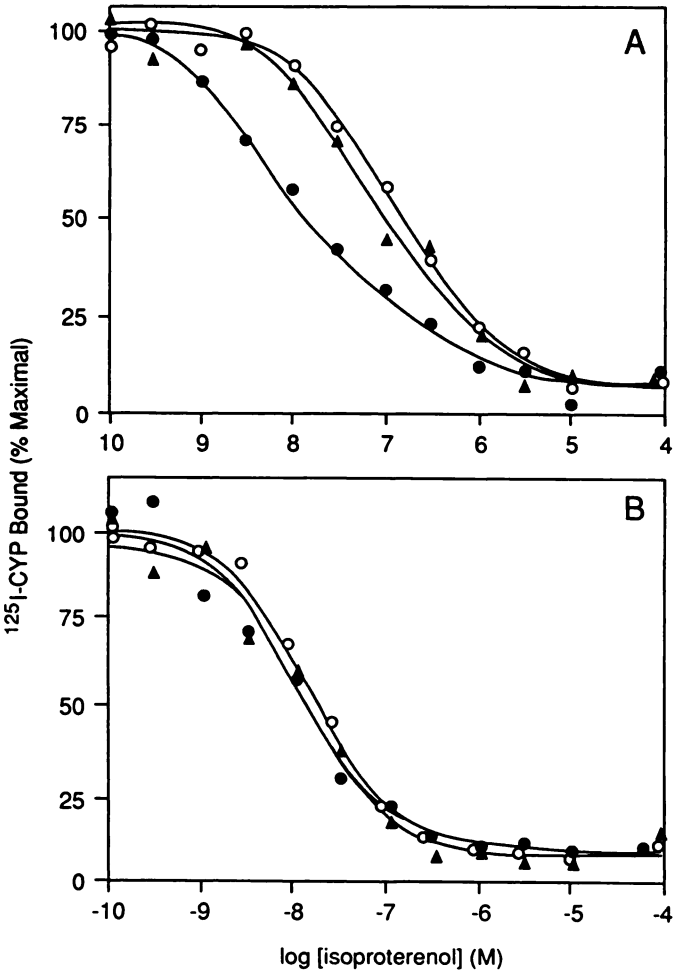


Fig. 4. Effects of L-451,167 on agonist binding to the wild-type and mutant βAR. Competition binding of the agonist isoproterenol to L cell membranes containing wild-type βAR (A) or D(222–229)βAR (B) was performed in the presence of 35 pM ^{125}I-CYP for 60 min at 23°, as described in Materials and Methods. Binding was measured in the absence (●) or presence of 100 μM Gpp(NH)p (○) or 50 μM L-451,167 (▲).

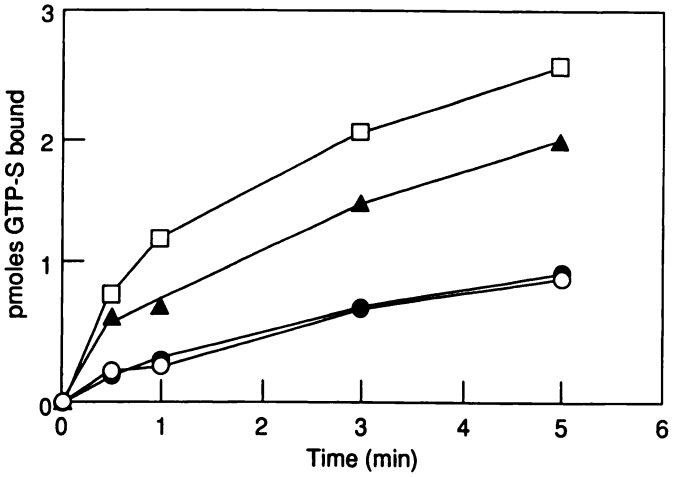


Fig. 5. Effects of L-451,167 and mastoparan on GTPγS binding to G proteins. A mixture of purified G_i and G_o was reconstituted into phospholipid vesicles and [^{35}S]GTPγS binding was measured as described in Materials and Methods. *Symbols* indicate G protein alone (●) or G protein in addition to 50 μM L-451,167 (○), 50 μM mastoparan (□), or both 50 μM mastoparan and 50 μM L-451,167 (▲). The rates of GTPγS binding for the four samples were: control, 0.18 ± 0.03 min^{-1}; + L-451,167, 0.16 ± 0.002 min^{-1}; + mastoparan, 0.63 ± 0.02 min^{-1}; + mastoparan and L-451,167, 0.49 ± 0.01 min^{-1}. The maximal equilibrium binding of GTPγS, measured at 30 min, was 2.56 pmol and was not affected by the addition of either mastoparan or L-451,167.

The decrease in agonist affinity observed in the presence of L-451,167 was similar to that observed in the presence of the nonhydrolyzable GTP analog Gpp(NH)p, and the effects of these two compounds were not additive. Both L-451,167 and Gpp(NH)p act to increase the proportion of α_2AR in the low affinity, rapidly dissociating agonist binding state, from 20–30% of the receptor in untreated membrane preparations to 70–80% in the presence of the compound, as assessed by both kinetic and equilibrium binding experiments. These data suggest that L-451,167 and GTP decrease agonist binding affinity via similar mechanisms, i.e., through uncoupling of the receptor from the G protein.

The uncoupling of receptors from G proteins by L-451,167 could reflect an interaction of the compound either with the receptor or with the G protein. The inhibition of mastoparan-stimulated [^{35}S]-GTPγS binding to G_i/G_o by L-451,167 suggests that this compound also interferes with the coupling of mastoparan to G proteins. Whether this inhibition results from an interaction of L-451,167 with mastoparan itself or with the mastoparan-binding site on the G protein remains to be determined. The compound does not affect the rate or level of GTPγS binding, indicating that it does not interact at the GTP binding site of the G protein. An interaction of this compound with other sites on the G protein cannot be excluded at present.

At the concentrations at which it uncouples the receptor from the G protein (1–50 μM), L-451,167 does not appear to be acting at the ligand binding site of the receptor, because it has no effect on the binding of antagonists in that concentration range. At higher concentrations (>100 μM), inhibition of binding of the antagonist ^{125}I-CYP to the βAR was observed, however, and inhibition of antagonist binding to the α_2AR was observed at concentrations of >150 μM. Further evidence that the action of L-451,167 in decreasing the affinity of these receptors for agonists does not result from an interaction at the ligand binding site arises from the lack of an effect of the compound on the affinity of the mutant D(222–229)βAR for agonists. This mutant receptor binds agonists and antagonists normally but fails to couple to G_s (11). Hence, any effect of L-451,167 on agonist binding to D(222–229)βAR would have suggested a direct interaction of the compound with the ligand binding site of the receptor. The fact that neither L-451,167 nor Gpp(NH)p decreases the affinity of D(222–229)βAR for agonists suggests that the reduction in agonist affinity for the wild-type receptor in the presence of L-451,167 reflects its uncoupling from G_s and that, at concentrations of <50 μM, the compound is not a direct inhibitor of ligand binding.

One hypothesis that would be consistent with these data is that L-451,167 acts directly at the site of receptor-G protein coupling to prevent the interaction between the two proteins. Site-directed mutagenesis of G-protein-coupled receptors has suggested that regions in the third intracellular loop, which would be predicted to form amphipathic α-helical extensions of the fifth and sixth transmembrane domains, may be involved in coupling to G proteins. The region of the G protein that interacts with the receptor has not been defined, but studies of chimeric G proteins suggest that the C-terminus of the protein

may be involved in this function (22). In addition, modification of a Cys residue in this domain of G_i and G_o by pertussis toxin-mediated ADP ribosylation impairs the coupling of the G protein with receptors (23). Modeling studies predict that the immediate C-terminal domain of the G protein could also form an amphipathic α-helix (24).

The amphipathic polyanionic characteristics of L-451,167 and heparin would be consistent with the intercalation of these compounds into an amphipathic α-helix on either the receptor or the G protein as a possible mechanism for their activity in uncoupling the interactions between these two proteins. Direct biophysical measurements of L-451,167 and purified receptors or G proteins will be necessary to characterize this site of interaction. Identification of the mechanism by which L-451,167 and heparin uncouple receptors from G proteins should provide insights into the biochemical basis for the interaction between these two proteins and further define a mechanism for potential therapeutic intervention.

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References

1. Gilman, A. G. G-proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–624 (1987).
2. Dohlman, H. G., M. G. Caron, and R. J. Lefkowitz. A family of receptors coupled to guanine nucleotide regulatory proteins. *Biochemistry* **26**:2657–2664 (1987).
3. Dixon, R. A. F., I. S. Sigal, and C. D. Strader. Structure-function analysis of the β-adrenergic receptor. *Cold Spring Harbor Symp. Quant. Biol.* **53**:487–489 (1989).
4. Findlay, J. B. C., and D. J. C. Pappin. The opsin family of proteins. *Biochem. J.* **238**:625–642 (1986).
5. Dixon, R. A. F., I. S. Sigal, E. Rands, R. B. Register, M. R. Candelore, A. D. Blake, and C. D. Strader. Ligand binding to the β-adrenergic receptor involves its rhodopsin-like core. *Nature (Lond.)* **326**:73–77 (1987).
6. Dixon, R. A. F., I. S. Sigal, M. R. Candelore, R. B. Register, W. Scattergood, E. Rands, and C. D. Strader. Structural features required for ligand binding to the β-adrenergic receptor. *EMBO J.* **6**:3269–3275 (1987).
7. Strader, C. D., I. S. Sigal, R. B. Register, M. R. Candelore, E. Rands, and R. A. F. Dixon. Identification of residues required for ligand binding to the β-adrenergic receptor. *Proc. Natl. Acad. Sci. USA* **84**:4384–4388 (1987).
8. Strader, C. D., I. S. Sigal, M. R. Candelore, E. Rands, W. S. Hill, and R. A. F. Dixon. Conserved aspartic acid residues 79 and 113 of the β-adrenergic receptor have different roles in receptor function. *J. Biol Chem.* **263**:10267–10271 (1988).
9. Strader, C. D., M. R. Candelore, W. S. Hill, I. S. Sigal, and R. A. F. Dixon. Identification of two serine residues involved in agonist activation of the β-adrenergic receptor. *J. Biol. Chem.* **264**:13572–13578 (1989).
10. Strader, C. D., R. A. F. Dixon, A. H. Cheung, M. R. Candelore, A. D. Blake, and I. S. Sigal. Mutations that uncouple the β-adrenergic receptor from G_s and increase agonist affinity. *J. Biol. Chem.* **262**:16439–16443 (1987).
11. Cheung, A. H., I. S. Sigal, R. A. F. Dixon, and C. D. Strader. Agonist-promoted sequestration of the β_2-adrenergic receptor requires regions involved in functional coupling with G_s. *Mol. Pharmacol.* **34**:132–138 (1989).
12. O'Dowd, B. F., M. Hnatowich, J. W. Regan, W. M. Leader, M. G. Caron, and R. J. Lefkowitz. Site-directed mutagenesis of the cytoplasmic domains of the human β_2-adrenergic receptor. *J. Biol. Chem.* **263**:15985–15992 (1988).
13. Kobilka, B. K., T. S. Kobilka, K. Daniel, J. W. Regan, M. G. Caron, and R. J. Lefkowitz. Chimeric α_2-,β_2-adrenergic receptors: delineation of domains involved in effector coupling and ligand binding specificity. *Science (Wash. D. C.)* **240**:1310–1316 (1988).
14. Kubo, T., H. Bujo, I. Akiba, J. Nakai, M. Mishina, and S. Numa. Location of a region of the muscarinic acetylcholine receptor involved in selective effector coupling. *FEBS Lett.* **241**:119–125 (1988).
15. Willuweit, B., and K. Aktories. Heparin uncouples α_2-adrenoceptors from the G_i-protein in membranes of human platelets. *Biochem. J.* **249**:857–863 (1988).
16. Butler, S. J., E. Kelly, F. McKenzie, S. Guild, M. Wakelam, and G. Milligan. Differential effects of suramin on the coupling of receptors to individual species of pertussis toxin sensitive guanine nucleotide binding proteins. *Biochem. J.* **251**:201–205 (1988).
17. Kobilka, B. K., H. Matsui, T. S. Kobilka, T. L. Yang-Feng, U. Francke, M. G. Caron, R. J. Lefkowitz, and J. W. Regan. Cloning, sequencing, and expression of the gene coding for the human platelet α_2-adrenergic receptor. *Science (Wash. D. C.)* **238**:650–656 (1987).
18. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand binding systems. *Anal. Biochem.* **107**:220–239 (1980).
19. Salomon, Y., C. Londos, and M. Rodbell. A highly sensitive adenylate cyclase assay. *Anal. Biochem.* **58**:541–548 (1974).
20. Northup, J. K., M. Smigel, and A. G. Gilman. The guanine nucleotide activating site of the regulatory component of adenylate cyclase. *J. Biol. Chem.* **257**:11416–11423 (1982).
21. Higashijima, T., S. Uzu, T. Nakajima, and E. M. Ross. Mastoparan, a peptide from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G-proteins). *J. Biol. Chem.* **262**:6491–6494 (1988).
22. Sullivan, K. A., R. T. Miller, S. B. Masters, B. Beiderman, W. Heideman, and H. R. Bourne. Identification of receptor contact site involved in receptor-G protein coupling. *Nature (Lond.)* **330**:758–760 (1987).
23. Sullivan, K. A., Y. C. Liao, A. Alborzi, B. Biederman, F. H. Chang, S. B. Masters, A. D. Levinson, and H. R. Bourne. Inhibitory and stimulatory G-proteins of adenylate cyclase: cDNA and amino acid sequences of the chains. *Proc. Natl. Acad. Sci. USA* **83**:6687–6691 (1986).
24. Masters, S. B., R. M. Stroud, and H. B. Bourne. Family of G-protein chains: amphipathic analysis and predicted structure of functional domains. *Protein Engineering* **1**:47–54 (1986).
25. Duggleby, R. G. Regression analysis of nonlinear Arrhenius plots: an empirical model and a computer program. *Comput. Biol. Med. Sci.* **14**:447–455 (1984).

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