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Allosteric Effects of G Protein Overexpression on the Binding of β-Adrenergic Ligands with Distinct Inverse Efficacies

MOUNIA AZZI, GRACIELA PIÑEYRO, STÉPHANIE PONTIER, STÉPHANE PARENT,[1] HERVÉ ANSANAY, AND MICHEL BOUVIER

Département de Biochimie and le Groupe de Recherches sur le Système Nerveux Autonome, Université de Montréal, Montréal, Québec, Canada

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ABSTRACT

Allosteric models of G protein-coupled receptors predict that G protein influences the spontaneous isomerization between inactive (R) and active (R*) conformations. Since inverse agonists have been proposed to preferentially bind to the inactive and uncoupled form(s), changes in the G protein content should influence the binding properties of these ligands. To test this hypothesis, we systematically assessed the effect of G proteins on the binding of β_2-adrenergic ligands with distinct levels of inverse efficacy. Recombinant baculoviruses encoding the human β_2-adrenoreceptor (β_2AR) were expressed alone or in combination with G protein subunits in Sf9 cells. Coexpression with the G protein $\alpha s\beta 1\gamma 2$ did not influence the relative efficacy of the ligands to inhibit the adenylyl cyclase but induced considerable decrease in number of sites detected by [^{3}H]ICI 118551, [^{3}H]propranolol, and ^{125}I-cyanopindolol. This loss was proportional to the inverse efficacy of the ligand used as the radiotracer in the assay. The addition of Gpp(NH)p inhibited the effects of G protein overexpression indicating that the G proteins acted allosterically. Consistent with this notion, Western blot analysis revealed that coexpression with the G proteins was not accompanied by a loss of immunoreactive β_2AR. Such allosteric effects of the G proteins were also observed in mammalian cells expressing endogenous level of G proteins indicating that the phenomenon is not unique to overexpression systems. Taken together, these results demonstrate that the apparent receptor number detected by radiolabeled inverse agonists is affected by the content in G proteins as a result of their influence on R/R* isomerization.

Traditionally, G protein-coupled receptors (GPCR) were considered to rest in an inactive conformation under basal conditions, requiring the presence of an agonist to undergo the necessary changes leading to activation and therefore receptor/G protein coupling. In this occupational interpretation of receptor activation, antagonists were believed to lack intrinsic activity, occupying the receptor without inducing changes in G protein coupling. More recently, a growing body of evidence has challenged this notion. Indeed, following the development of receptor mutants that show constitutive (ligand-independent) activity and that of overexpression systems that revealed spontaneous agonist-independent activity even for wild-type GPCRs, the notion that some ligands (inverse agonists) may inhibit constitutive/spontaneous activity has emerged (for review, see Kenakin, 1996; Bond and Bouvier, 1998). As traditionally described for agonists, inverse agonists were also found to display different efficacies, ranging from almost neutral antagonism to full inverse agonism (Chidiac et al., 1994; Leeb-Lundberg et al., 1994; Labrecque et al., 1995; Gardella et al., 1996; Mullaney et al., 1996; Smith et al., 1996; Lee et al., 1997; Jansson et al., 1998). Based on the interpretation of these studies, numerous thermodynamic models proposing the allosteric interconversion of receptors between active and inactive states have been proposed (Samama et al., 1993; Kenakin, 1995; Weiss et al., 1996; Leff and Scaramellini, 1998).

According to the two-state model (Karlin, 1967; Colquhoun, 1973; Thron, 1973; Leff, 1995), agonist-independent activation of GPCRs is believed to reflect the spontaneous isomerization of the receptor between inactive (R) and active (R*) conformations, with the equilibrium under native conditions shifted toward R for most receptors. In this model, agonists are considered as preferentially binding and stabilizing R* (thus leading to activation) whereas inverse agonists by binding to R would inhibit the system. Neutral

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[1] Present address: Biosignal Inc., 1744 William Street, Montréal, H3J 1R4 Canada.

ABBREVIATIONS: GPCRs, G protein-coupled receptors; β_2AR, β_2-adrenergic receptor; CAM, constitutively activated mutant; Sf9, *Spodoptera frugiperda* 9; G protein, guanine nucleotide-binding protein; Gpp(NH)p, guanylylimidodiphosphate; PBS, phosphate buffer saline; ICI 118551, *erythro*-DL-1-(7-methylindan-4,1-oxy)-3-isopropylaminobutan-2-ol; HEK, human embryonic kidney; PTX, pertussis toxin; CTX, cholera toxin; FACS, fluorescence-activated cell sorter; PAGE, polyacrylamide gel electrophoresis; m.o.i., multiplicity of infection.

antagonists that do not discriminate between the two conformers would leave the equilibrium between the two isomers unchanged. Furthermore, receptors are known to interact with a transducer G protein and thus to exist in an uncoupled (R) and a coupled (RG) conformation. Based on the thermodynamic description of the ternary complex model and its extended form (De Lean et al., 1980; Lefkowitz et al., 1993; Samama et al., 1993), R* would spontaneously couple to the G protein whereas the interaction between inactive R and G would not be favored. Furthermore, the activated R* state has been shown to be stabilized by the guanine nucleotide-free G protein α-subunit (Seifert et al., 1998), and it is this activated/coupled form (R*G) of the receptor that displays the highest affinity for agonists. On the other hand, GTP analogs are considered to decrease the affinity of agonists for their cognate receptors by promoting receptor/G protein dissociation (De Lean et al., 1980; Kenakin, 1996).

In keeping with the prediction of the two-state allosteric model, it is expected that any condition that would change the equilibrium between R and R* must have an impact on receptor activity and ligand affinity simultaneously. In keeping with this prediction, it has been shown for many GPCRs that an increase in constitutive activity is linked to an increase in apparent agonist affinity (Cotecchia et al., 1990; Kjelsberg et al., 1992; Ren et al., 1993; Samama et al., 1993) and that such increases in binding affinity correlate well with the pharmacological efficacy of agonists (Samama et al., 1993). On the other hand, if as predicted by the two-state model, inverse agonists bind preferentially to the inactive form of the receptor, the apparent affinity of these drugs is expected to decrease in conditions that shift the equilibrium toward R* (Gether and Kobilka, 1998). Although changes in inverse agonist binding have been reported in some studies in which R/R* or R/RG equilibrium was manipulated (Barker et al., 1994; Bouaboula et al., 1997; Francken et al., 2000), no study has systematically examined the prediction of the two-state model on inverse agonist binding properties.

The aim of the present study was to directly assess the effect of promoting R*G formation on inverse agonist binding. For this purpose, we took advantage of the baculovirus/*Spodoptera frugiperda* 9 (Sf9) cell expression system, which has extensively been used to reconstitute the interactions between specific GPCRs and their cognate G proteins (Butkerait et al., 1995; Grunewald et al., 1996; Barr and Manning, 1997; Barr et al., 1997; Francken et al., 2000). Overexpression of Gαsβ1γ2 led to a significant reduction of inverse agonist binding to the human β_2AR expressed in the same cells. In conformity with the two-state model, the extent of the changes in inverse agonist binding was proportional to inverse efficacy of the ligands tested. Similar binding decreases were also observed when a constitutively active β_2AR mutant was used, indicating that stabilization of R* through either constitutively activating mutation or overexpression of G protein has similar effects on inverse agonist binding properties.

Materials and Methods

Baculoviruses. The recombinant *c-myc* β_2AR human baculoviruses was constructed by Mouillac et al. (1992). Recombinant baculoviruses encoding the different G protein subunits (rat αs short form, rat αi_1, human αq, bovine β1, bovine γ2) and β-galactosidase were obtained from Biosignal (Montréal, Québec). Those encoding the constitutively active mutant β_2AR were kindly provided by Dr. B. K. Kobilka (HHMI, Stanford University Medical School, CA).

Cell Culture and Baculovirus Infection. Sf9 cells were cultured as monolayers in T flasks as described previously (Chidiac et al., 1996). Cells (at a density of 1–2 $\times$ 10^6 cells/ml) were infected with *c-myc* β_2AR or constitutively active mutant β_2AR encoding baculovirus at a multiplicity of infection (m.o.i.) of 1. For the coexpression of multiple Gα- (Gαs, Gαi1, and Gαq), Gβ1-, and Gγ2-subunits, an m.o.i. of 2 was used for each virus. The total m.o.i. was maintained at 8 in all infections by using the appropriate amount of β-galactosidase virus. At 48-h postinfection, cells were harvested by centrifugation, washed twice with ice-cold phosphate-buffered saline (PBS), and directly used for membrane preparation. HEK293s cells stably expressing the β_2AR were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1 mM glutamine, 500 units/ml penicillin, and 500 units/ml streptomycin.

Membrane Preparation. Cells were resuspended and lysed under hypotonic conditions (5 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 μg/ml leupeptine, 5 μg/ml soybean trypsin inhibitor, and 10 μg/ml benzamidine) and homogenized with a polytron homogenizer (Ultra-Turrax; Janke and Kunkel, Staufen, Germany) for 5 s. Homogenates were centrifuged at 500*g* for 5 min at 4°C, and the resulting supernatant fraction was centrifuged at 25,000*g* for 20 min at 4°C. The membrane pellets were washed twice in the same buffer and centrifuged under the same conditions. The membrane pellet was finally resuspended in 75 mM Tris-HCl, pH 7.4, containing 5 mM $MgCl_2$, 2 mM EDTA, and protease inhibitors as in the lysis buffer. The membranes were immediately used for adenylyl cyclase activity and radioligand binding assay or frozen at −80°C for Western blot analysis. Protein content in membrane preparation was estimated with the Bradford protein assay (Bradford, 1976) using the Bio-Rad kit. Bovine serum albumin was used as a standard.

Adenylyl Cyclase Activity. Membrane adenylyl cyclase activity was performed according to the method of Salomon et al. (1974). Briefly, 5 μg of membrane protein was incubated in the absence or presence of various concentrations of β_2AR ligands with 0.12 mM ATP, 10^6 cpm [α-^{32}P]ATP/assay (NEN Mandel), 0.1 mM cAMP, 53 μM GTP, 2.7 mM phosphoenolpyruvate, 0.1 mM isobutylmethylxanthine, 1 unit of myokinase, and 0.2 unit of pyruvate kinase, in a total volume of 50 μl. The samples were incubated at 37°C for 15 min, and the reaction was stopped by addition of 1 ml of a cold solution containing 0.4 mM ATP, 0.3 mM cAMP, and [^{3}H]cAMP (25,000 cpm). cAMP was isolated by sequential chromatography using a Dowex gel followed by aluminum oxide.

Radioligand Binding Assay. Radioligand binding assays were performed essentially as described previously (Chidiac et al., 1996). Briefly, 1 to 3 μg of membrane proteins were incubated in a total volume of 0.5 ml in a buffer containing 75 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, protease inhibitors, and varying concentrations of the different radioligands tested: ^{125}I-cyanopindolol (NEN Mandel), [^{3}H]propranolol (NEN Mandel), or [^{3}H]ICI 118551 (Tocris Cookson, St. Louis, MO). For single-point binding analysis, saturating concentrations of each ligand were used: 250 pM, 6 nM, and 8 nM for ^{125}I-cyanopindolol, [^{3}H]propranolol, and [^{3}H]ICI 118551, respectively. Nonspecific binding was estimated in the presence of 10 μM alprenolol (Sigma, St. Louis, MO). Binding reactions were incubated at room temperature for 60 min for [^{3}H]propranolol and 90 min for ^{125}I-cyanopindolol and [^{3}H]ICI 118551 and were terminated by rapid filtration through glass fiber (GF/C) filters (Whatman, Maidstone, UK) with ice-cold 25 mM Tris-HCl, pH 7.4. The experiments with Gpp(NH)p were performed with a concentration of 100 μM in the binding assay.

Western Blot Analysis. Membranes from Sf9 cells expressing the β_2AR and different G protein subunits were reduced in Laemmli's sample buffer (Laemmli, 1970) and 5 and 10 μg of proteins were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred to nitrocellulose membrane (Xymotech) and

probed for 1 h at room temperature with a mouse anti-*c-myc* monoclonal antibody (9E10, 1:2,000), a rabbit anti-β_2AR polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:10,000), and rabbit anti-Gs, Gi, or Gq polyclonal antibodies (Santa Cruz Biotechnology, Inc.; 1:5,000). The first antibody was revealed with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ; 1:20,000) and chemiluminescence using Renaissance plus kit (NEN Mandel). To ensure that the intensity of the signal was directly proportional to the amount of receptor protein loaded, serial dilutions of each membrane preparation were analyzed.

Flow Cytometry. The flow cytometry was performed as previously described (Morello et al., 2000). Briefly, Sf9 cells were permeabilized in PBS containing 0.15% Triton, fixed with 3% paraformaldehyde, and subsequently incubated with anti-β_2AR (Santa Cruz Biotechnology, Inc.) antibody for 1 h at room temperature. The cells were then washed with PBS and incubated with phycoerythrin-conjugated goat anti-rabbit antibody (Immunotech, Westbrook, ME) for 1 h at room temperature. The cells were washed in PBS and analyzed on a FACS caliber Becton-Dickson flow cytometer (BD Immunocytometry Systems, San Jose, CA) set up to detect phycoerythrin fluorescence (585 ± 21 nm). For each sample, 10,000 cells were analyzed.

Data Analysis. For adenylyl cyclase assay, the data were calculated as picomoles of cAMP produced per minute per milligram of proteins and were expressed as percentage of control. Dose-response curves and saturation experiments were analyzed by nonlinear regression using the Prism program (GraphPad Software, San Diego, CA). EC_{50} values were derived from the curves. Maximal efficacies of each drug were determined as previously defined (Chidiac et al., 1994). For each set of data, the efficacy of the most inhibitory ligand, ICI 118551, was set at −1. B_{max} and K_d values of the radioligand and IC_{50} values of inhibitors were derived from the curve fitting. Statistical analyses were performed using the Student's t test. $p < 0.05$ was considered statistically significant.

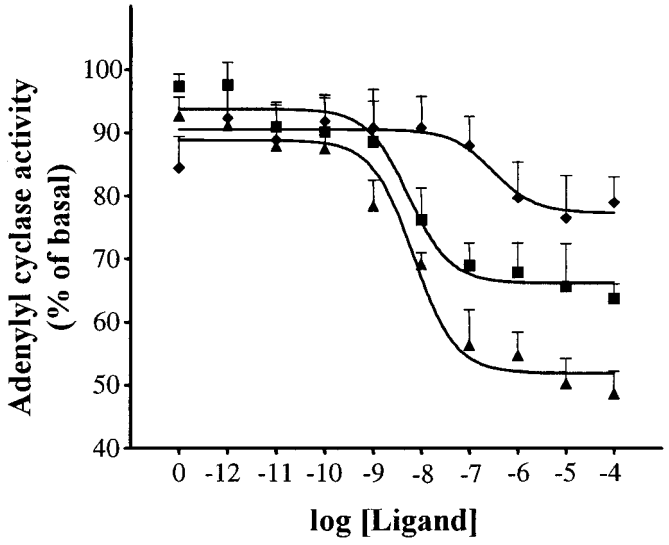


Fig. 1. Inhibition of spontaneous adenylyl cyclase activity in membranes derived from Sf9 cells expressing the human β_2AR. Adenylyl cyclase activity was determined as described under *Materials and Methods* in the presence of increasing concentrations (10^{-12}–10^{-4} M) of cyanopindolol (♦), propranolol (■), or ICI 118551 (▲). Values are expressed as percentage of ligand-independent (basal) activity and presented as the means ± S.E.M. of five to six independent experiments. Basal adenylyl cyclase activity ranged from 15.5 to 36.8 pmol of cAMP/mg of protein/min in these experiments. Mean EC_{50} and maximum inhibition values are summarized in Table 1.

Results

In an effort to systematically test the hypothesis that inverse agonists bind with higher affinity to the inactive/uncoupled forms of the receptor (R), we assessed the influence of G protein overexpression and constitutive activation of the β_2AR in direct radioligand binding experiments. For this purpose, the relative efficacy of commonly used β_2AR radioligands was first assessed in membranes from Sf9 cells expressing a high copy number of the human β_2AR (8.43 ± 1.76 pmol/mg of protein). As shown in Fig. 1, ICI 118551, propranolol, and cyanopindolol were found to be inverse agonists because they inhibited the basal adenylyl cyclase activity by 48, 34, and 23%, respectively. The level of adenylyl cyclase activity, detected at the highest ICI 118551 concentrations, was undistinguishable from that observed in cells that did not express the receptor (data not shown), and thus an efficacy of −1 (full inverse agonist) was attributed to this compound. The inverse efficacies of cyanopindolol and propranolol were expressed relative to that of ICI 118551 (Table 1).

Coexpression of the β_2AR with Gαsβ1γ2 promoted a 2.9-fold increase in the basal adenylyl cyclase activity (60 ± 0.6 versus 175 ± 13 pmol/mg of protein/min for membranes from Sf9 cells expressing the β_2AR and the β_2AR/Gαsβ1γ2,respectively) but did not influence the relative efficacy nor the potency of the three compounds tested (Table 1).

To test the effect of G protein coupling on the binding properties of these inverse agonists, saturation binding assays were carried out in membranes derived from cells expressing the receptor alone or in combination with Gαsβ1γ2. As shown in Fig. 2 and Table 2, the apparent affinities of ^{125}I-cyanopindolol, [^{3}H]propranolol, and [^{3}H]ICI 118551 for the β_2AR were not affected by the coexpression with Gαsβ1γ2, although a significant decrease in the B_{max} values was observed for the three inverse agonists tested (Fig. 2 and Table 2). To further document this effect of the G protein expression on the apparent binding capacity of the receptor, a large number of binding experiments were carried out with saturating concentration of each of the ligands. Figure 3 summarizes the results obtained in 12 to 15 independent

TABLE 1

Inverse efficacies and potencies of different β_2AR ligands assessed in membranes expressing the β_2AR alone or in combination with Gαsβ1γ2

E_{inv} and EC_{50} values were determined as described under *Materials and Methods*. Data shown represent the means of n independent experiments performed in duplicate. The efficacies of cyanopindolol and propranolol were scaled with ICI 118551 efficacy set at −1.00. Numbers into brackets represent the minimum and maximum for each condition.

Ligand	E_{inv}		EC_{50} (nM)	
	β_2AR	β_2AR/αsβ1γ2	β_2AR	β_2AR/αsβ1γ2
n	(5–6)	(2)	(5–6)	(2)
Cyanopindolol	−0.37 (−0.26; −0.45)	−0.46 (−0.39; −0.53)	301 (92.5; 783)	373 (190; 556)
Propranolol	−0.69 (−0.53; −0.83)	−0.69 (−0.57; −0.81)	5.3 (1.2; 24.1)	5.2 (2.5; 8.0)
ICI 118551	−1.00	−1.00	6.9 (2.2; 21.5)	10.4 (5.3; 15.5)

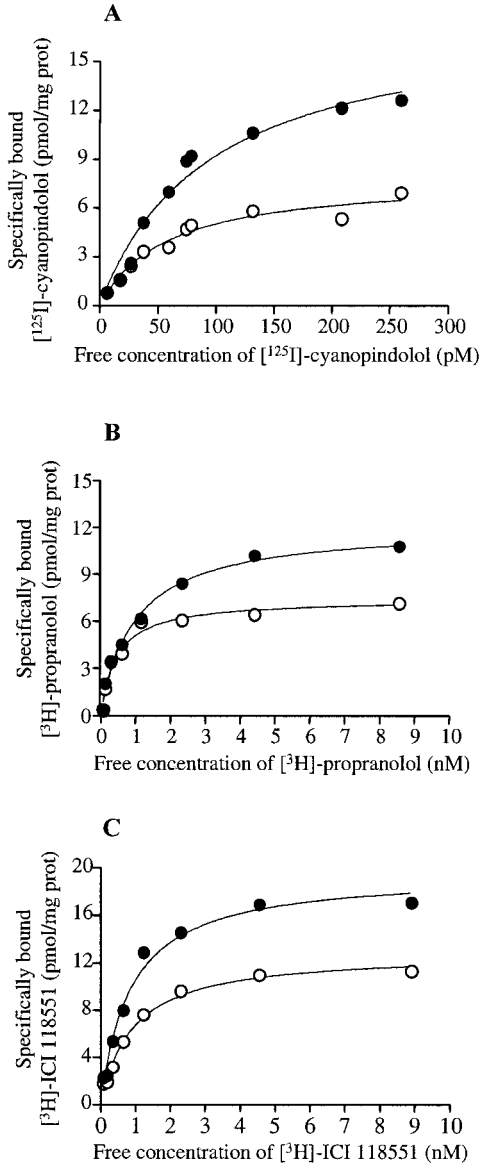


Fig. 2. Saturation binding isotherms for ^{125}I-cyanopindolol, [^{3}H]propranolol, and [^{3}H]ICI 118551. Membranes derived from Sf9 cells expressing the β_2AR alone (●) or in combination with Gαsβ1γ2 (○) were incubated in the presence of increasing concentration of radioligand and the specific binding determined as described under *Materials and Methods*. The data represent mean values of triplicate determinations from a typical experiment. Saturation curves were fitted to a one-binding site model using a least-squares nonlinear regression analysis (GraphPad). B_{max} and K_d values were derived for each individual experiment, and mean values of three to four independent experiments are summarized in Table 2.

experiments. Not only was the decrease in B_{max} promoted by the coexpression of Gαsβ1γ2 found to be highly reproducible, but the extent of the reduction was proportional ($r^2 = 0.989$) to the relative inverse efficacy of the ligand used in the binding assay (Fig. 3B).

The loss in binding sites observed did not result from a reduction in β_2AR protein expression since an increase rather than a decrease in immunoreactive β_2AR was observed upon coexpression with Gαsβ1γ2 (Fig. 4). The increase in the amount of receptor protein detected most likely results from a stabilizing effect of the G protein. This effect cannot be attributed to a particularity of the N terminus *myc* epitope tag since similar results were obtained using an anti-β_2AR antibody directed against the carboxyl tail of the receptor (Santa Cruz Biotechnology, Inc.). Indeed, both the 45- to 55-kDa bands corresponding to heterogeneously glycosylated monomeric forms of the receptor and the dimeric species observed at ~120 kDa were found to be increased using the two antibodies. The detection of a greater number of bands in the 45- to 55-kDa region when using the anti-β_2AR could be due to the fact that the *myc* epitope is localized close to the N terminus glycosylation site where it may be partly masked by the carbohydrate moieties, whereas the anti-β_2AR is directed against the C terminus of the receptor.

The apparent contradiction between the loss of binding site and the increase in receptor protein suggests that only a fraction of the receptor population can bind the radioligands used in the assays. This proposition would be consistent with the idea that the increased expression of G proteins stabilizes a coupled/activated state of the receptor that has an affinity for the inverse agonists that is too low to be detected in the binding assay.

If the latter hypothesis is true, one would predict that promoting the uncoupling of the receptor from the G protein by adding guanine nucleotide in the binding assay would reduce the effect of the G protein overexpression. As can be seen in Fig. 5, the addition of Gpp(NH)p in the binding assay reduced the loss of binding sites detected with [^{3}H]ICI 118551, [^{3}H]propranolol, and ^{125}I-cyanopindolol upon coexpression of Gαsβ1γ2.

To test the specificity of the phenomenon, coexpression with another G protein that has been shown to interact with the β_2AR, Gi (Daaka et al., 1997), and one that does not, Gq (Offermanns and Simon, 1995), was investigated. As for Gαsβ1γ2, coexpression of the receptor with G$\alpha i_1\beta$1γ2 led to a significant reduction of the number of sites that were recognized by the three inverse agonists (Fig. 6A). In contrast, coexpression with Gαqβ1γ2 was without effect. Since Gαq

TABLE 2

Binding parameters of ^{125}I-cyanopindolol, [^{3}H]propranolol, and [^{3}H]ICI 118551 derived from saturation experiments carried out in membranes expressing the β_2AR alone or in combination with Gαsβ1γ2

K_d and B_{max} were determined as described under *Materials and Methods*. Data were analyzed using the curve-fitting software GraphPad-Prism. Values represent means ± S.E.M. of n independent experiments performed in triplicates.

Ligand	n	K_d		B_{max}	
		β_2AR	β_2AR/Gαsβ1γ2	β_2AR	β_2AR/Gαsβ1γ2
				pmol/mg protein	
Cyanopindolol	4	94 ± 19 pM	84 ± 23 pM	18 ± 2.15	11 ± 0.39[a]
Propranolol	3	0.9 ± 0.31 nM	0.7 ± 0.26 nM	14 ± 0.56	8.6 ± 1.00[b]
ICI 118551	4	1.3 ± 0.17 nM	1.3 ± 0.37 nM	17 ± 1.78	8.4 ± 1.40[b]

[a] $p < 0.05$.
[b] $p < 0.01$.

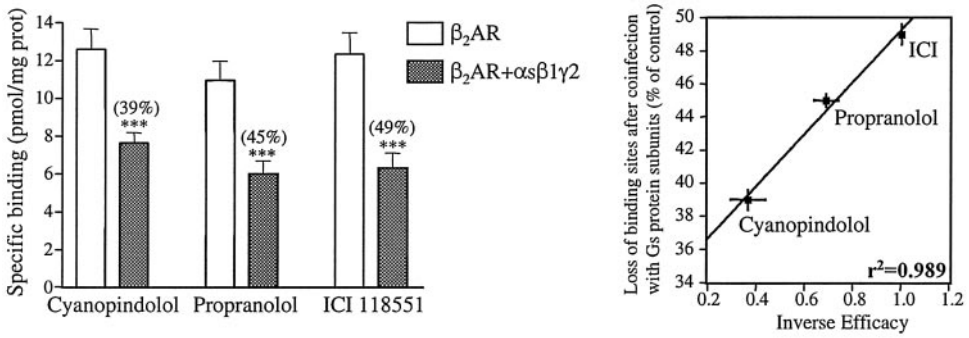
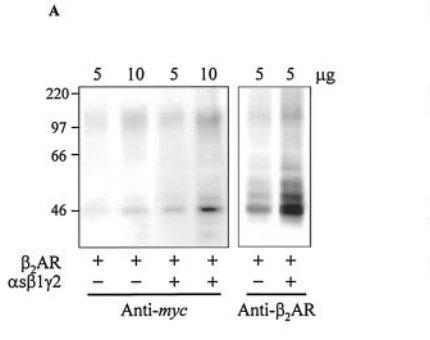


Fig. 3. Effect of Gαsβ1γ2 expression on the binding of [^{3}H]ICI 118551, [^{3}H]propranolol, and ^{125}I-cyanopindolol to β_2AR. Sf9 cells were infected with the β_2AR encoding baculovirus alone or in combination with baculovirus encoding Gαsβ1γ2. The number of binding sites detected by saturating concentrations of [^{3}H]ICI 118551 (n = 12), [^{3}H]propranolol (n = 12) and ^{125}I-cyanopindolol (n = 15) were then assessed in membrane preparations by direct radioligand binding assays as described under *Materials and Methods.* A, data are expressed as picomoles of receptor per milligram of membrane protein. The numbers in brackets represent the percentage of loss of binding sites. B, correlation between the E_{inv} values and the loss of binding sites detected by each ligand upon expression of the G protein subunits. Statistical significance of the differences were assessed using the paired two-tailed Student's t test; ***, $p < 0.001$.

could readily be detected by Western blot analysis (Fig. 6B), the data indicate that the effect of G proteins on inverse agonist binding is linked to their selectivity of coupling to the receptor. However, the similar effect observed for Gαs and Gαi cannot be interpreted as an equivalent affinity of the receptor for the two G proteins since their relative level of expression cannot be easily determined. Nevertheless, to determine whether the influence of the G proteins can be observed at physiological level of expression, the effect of uncoupling the G proteins from the receptor using either cholera (CTX) or pertussis (PTX) toxins was investigated in HEK293s cells stably expressing the β_2AR. As shown in Fig. 6C, a significant increase in ^{125}I-cyanopindolol binding capacity was observed upon treatment with both toxins indicating that coupling to the two G proteins occurs at endogenous level of expression and can influence receptor binding properties.

In an effort to determine which of the α-subunit, the $\beta\gamma$-dimer, or the heterotrimer is responsible for the reduction in binding sites detected by the inverse agonists, the number of receptors measured by ^{125}I-cyanopindolol was assessed following coexpression with Gαs, Gβ1γ2, or Gαsβ1γ2. A significant reduction was observed in the three coexpression conditions (Fig. 7). However, the greatest reduction was seen upon coexpression with the heterotrimer, 43%, compared with 34% and 17% for coexpression with Gαs and Gβ1γ2, respectively. Given that exogenous Gαs and Gβ1γ2 can interact with the insect αs-like and $\beta\gamma$-subunits, these data suggests that interaction with the heterotrimer is most likely responsible for G protein effect on inverse agonist binding.

If the assumption that the loss of inverse agonist binding sites results from a stabilization of the activated form of the receptor by the heterotrimeric G protein is correct, one could predict that mutations that lead to constitutive activation of the receptor should have similar effects. To test this hypothesis, ^{125}I-cyanopindolol binding was assessed in cells expressing either the wild-type or the constitutively active β_2AR (CAMβ_2AR) in which L266, K267, H269, and L272 were replaced by S, R, K, and A that correspond to the homologous region of constitutively active α1b adrenergic receptor (Cotecchia et al., 1990; Samama et al., 1993). When cells expressing identical amounts of either β_2AR or CAMβ_2AR protein, as assessed by FACS analysis (Fig. 8A), were tested for the number of ^{125}I-cyanopindolol binding



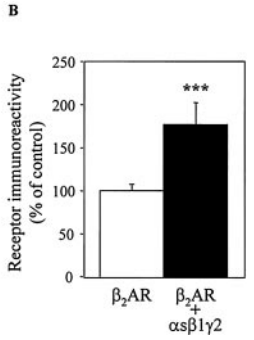


Fig. 4. Effect of Gαsβ1γ2 expression on the level of β_2AR protein detected by Western blot. Solubilized membrane proteins derived from Sf9 cells expressing *myc*-tagged β_2AR alone or in combination with Gαsβ1γ2 were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with 9E10 anti-myc antibody or anti-β_2AR antibody as described under *Materials and Methods.* A, 5 and 10 μg of protein were loaded for each condition. Numbers on the left indicate molecular weight of marker proteins. B, densitometric analysis of immunoreactive β_2AR expressed as the percentage of the value observed in cells expressing the β_2AR alone (control). Values represent the means ± S.E.M. of three independent experiments. Statistical significance of the differences were assessed using the paired two-tailed Student's t test; ***, $p < 0.001$.

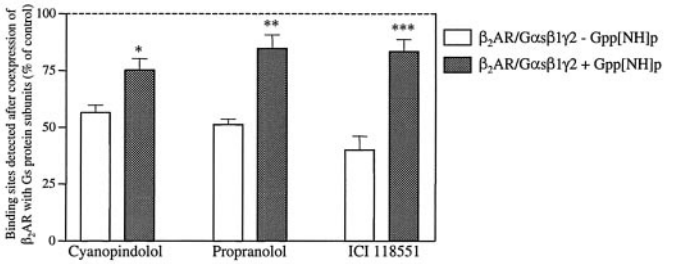


Fig. 5. Effect of Gpp(NH)p on inverse agonists binding. The number of binding sites detected by [^{3}H]ICI 118551 (n = 6), [^{3}H]propranolol (n = 5), and ^{125}I-cyanopindolol (n = 6) was assessed in membranes derived from cells expressing the β_2AR alone or in combination with Gαsβ1γ2 in the presence or absence of 100 μM Gpp(NH)p as described under *Materials and Methods*. Data are expressed as the percentage of binding sites detected by each radioligand in membranes expressing the β_2AR alone in the absence of Gpp(NH)p and represent the means ± S.E.M. of five to six independent experiments. The dotted line represents the number of sites detected in membrane expressing the β_2AR alone. Statistical significance of the differences was assessed using the paired two-tailed Student's t test; ***, $p < 0.001$.

sites, a significantly lower number of sites were found for the CAMβ_2AR (Fig. 8B). To confirm that the difference in radioligand binding did not result from a lower level of protein expression or greater degradation, Western blot analyses were carried out. Equivalent amounts of binding sites detected by ^{125}I-cyanopindolol in membranes expressing the wild-type β_2AR or the CAMβ_2AR were resolved by electrophoresis and detected using an anti-β_2AR antibody (Santa Cruz Biotechnology, Inc.). As shown in Fig. 8C, the total amount of β_2AR immunoreactivity was 1.5-fold higher in membranes from Sf9 cells infected with the CAMβ_2AR than those expressing the wild-type receptor, thus confirming that a significant proportion of the CAMβ_2AR is unable to bind ^{125}I-cyanopindolol.

To investigate whether CAMβ_2AR coupling to Gs protein could further modify inverse agonist binding, the specific binding of ^{125}I-cyanopindolol, [^{3}H]propranolol, and [^{3}H]ICI 118551 was assessed in membranes derived from Sf9 cells expressing the constitutively active receptor alone or in combination with Gαsβ1γ2. As previously observed for the wild-type β_2AR (Fig. 3A), the number of CAMβ_2AR binding sites detected by the three radioligands tested was significantly reduced upon overexpression of Gαs (Fig. 9), indicating that interaction with the G protein can further stabilize the CAMβ_2AR into a conformation that is not recognized by the inverse agonists.

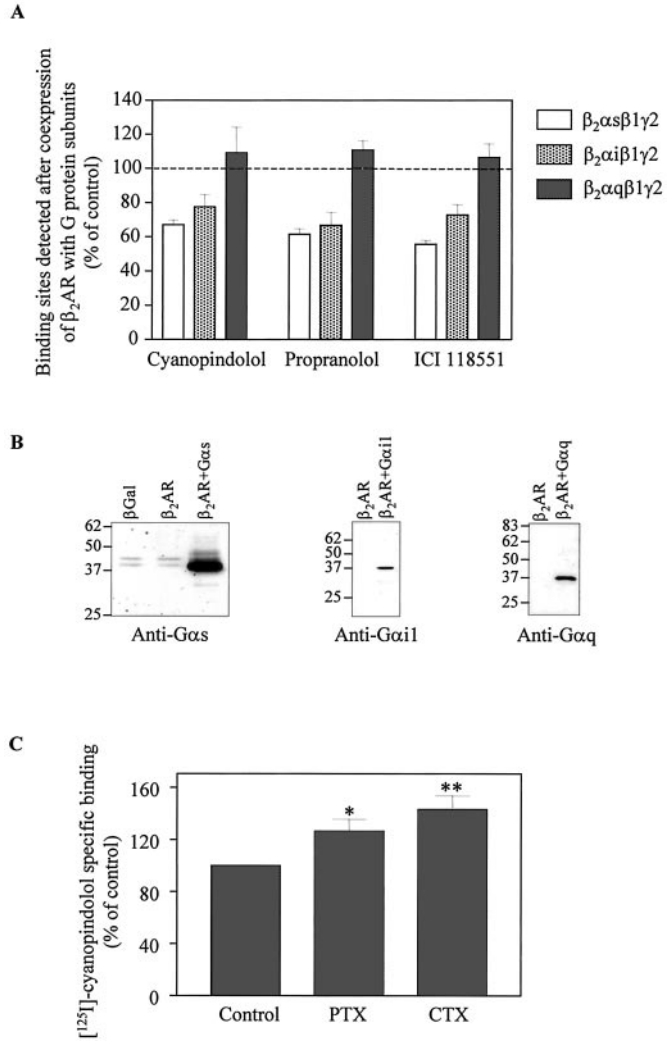


Fig. 6. Effect of G protein expression on the binding of inverse agonists to the β_2AR. A and B, Sf9 cells were infected with baculoviruses encoding the β_2AR alone or in combination with viruses encoding Gαsβ1γ2, G$\alpha i_1\beta$1γ2, or Gαqβ1γ2. A, [^{3}H]ICI 118551, [^{3}H]propranolol, and ^{125}I-cyanopindolol binding was assessed in membranes expressing the β_2AR alone or in combination with the indicated G protein subunits. Data are expressed as percentage of binding sites detected in membranes expressing the β_2AR alone and represent the means ± S.E.M. of four independent experiments for each radioligand. The dashed line represents the number of sites detected in membrane expressing the β_2AR alone. Statistical significance of the differences were assessed using the paired two-tailed Student's *t* test; ***, $p < 0.001$. B, the expression of each of the α-subunits was assessed by Western blot analysis using selective antibodies (see *Materials and Methods*). The faints bands detected with the anti-Gαs in cells transfected with either β-galactosidase (βGal) or β_2AR encoding viruses alone reflect the presence of endogenous long and short forms of the insect Gαs. C, ^{125}I-cyanopindolol binding was assessed in membrane preparation from HEK293s cells stably expressing the human β_2AR that were treated with PTX (50 ng/ml, 16 h) or CTX (300 ng/ml, 24 h) or were untreated. Data are expressed as the percentage of binding sites detected by the radioligand in control membranes expressing the β_2AR and represent the means ± S.E.M. of four independent experiments. Statistical significance of the differences were assessed using paired two-tailed Student's *t* test; *, $p < 0.05$; **, $p < 0.01$.

Discussion

The results of the present study demonstrate that overexpression of trimeric G proteins (G$\alpha s\beta_1\gamma_2$ or G$\alpha i\beta_1\gamma_2$) results in a significant decrease of the total number of β_2AR sites detected by inverse agonists. This finding is consistent with two of the predictions made by allosteric models for receptor activation (such as the extended ternary and the cubic models): 1) inverse agonists bind preferentially to the inactive (R) form of the receptor and 2) the interaction of the receptor with its cognate G protein stabilize the active form (R*) at the expense of R (Lefkowitz et al., 1993; Samama et al., 1993; Bond et al., 1995; Kenakin, 1995; Leff, 1995; Leff and Scaramellini, 1998). Similarly, our observation that the extent of decrease in inverse agonist binding is directly correlated to the inverse efficacy of the ligand used is consistent with efficacy-related changes in ligand binding predicted by all of these models following changes in R/R* ratio. Indeed, the

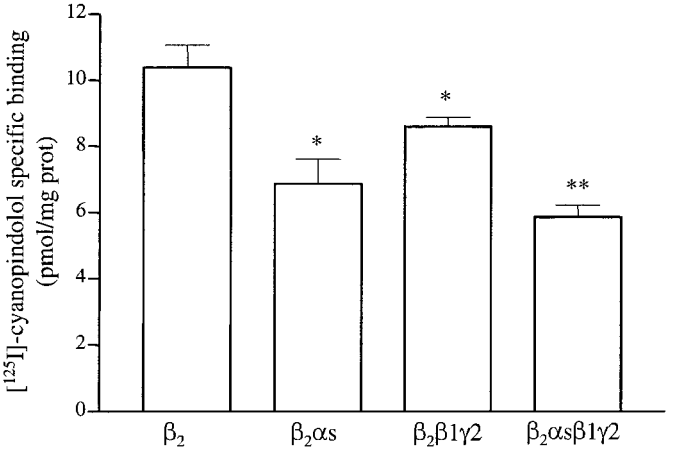


Fig. 7. Effect of Gαs, Gβ1γ2, and Gαsβ1γ2 expression on ^{125}I-cyanopindolol binding to the β_2AR. ^{125}I-Cyanopindolol binding was assessed in membranes expressing the β_2AR alone or in combination with the indicated G protein subunits. Data are expressed as picomoles of receptor per milligram of membrane protein and represent the means ± S.E.M. of three independent experiments for each condition. Statistical significance of the differences were assessed using the paired two-tailed Student's *t* test; ***, $p < 0.001$.

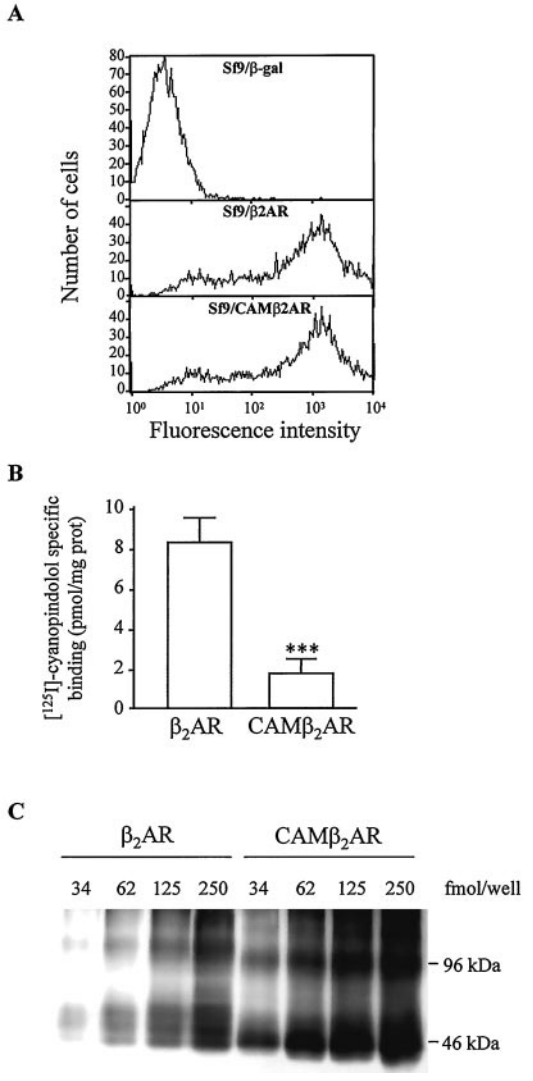


Fig. 8. Binding capacity of wild-type (WT) and constitutively activated mutant (CAM) β_2AR. A, WT and CAMβ_2AR total cell expression quantified by cytofluorometry (FACS) analysis as described under *Materials and Methods*. Data presented are typical of two independent experiments. B, binding assays using a saturating concentration of ^{125}I-cyanopindolol were carried out in membranes derived from cells expressing WT or CAMβ_2AR. Data are expressed as picomoles per milligram of protein and represent the means ± S.E.M. of five independent experiments for each of the receptor constructs. Statistical significance of the differences was assessed using the paired two-tailed Student's t test; ***, $p < 0.001$. C, solubilized membrane proteins derived from cells expressing WT or CAMβ_2AR were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with an antibody raised against the carboxyl-terminal tail of the β_2AR as described under *Materials and Methods*. Thirty-four to 250 fmol of receptor detected by ^{125}I-cyanopindolol was loaded for each construct.

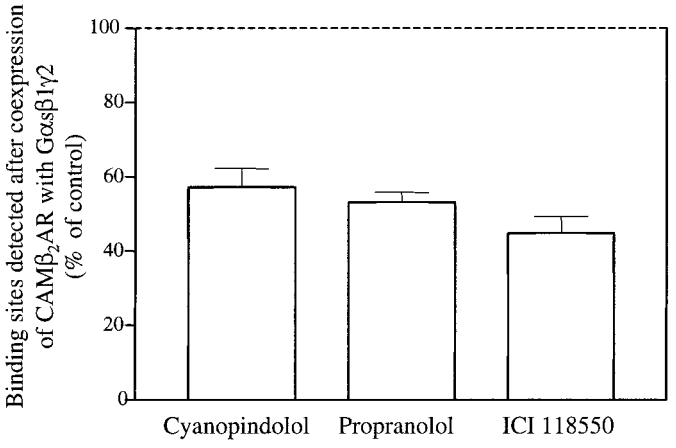


Fig. 9. Effect of Gαsβ1γ2 expression on the binding of [^{3}H]ICI 118551, [^{3}H]propranolol, and ^{125}I-cyanopindolol to CAMβ_2AR. Radioligand binding was carried out in membranes derived from cells expressing the CAMβ_2AR alone or in combination with Gαsβ1γ2 using saturating concentrations of the radioligands as described under *Materials and Methods*. Data are expressed as the percentage of binding sites detected in membranes expressing the CAMβ_2AR alone and represent the means ± S.E.M. of five independent experiments for each radioligand. The dashed line represents the number of sites detected in membrane expressing the β_2AR alone. Statistical significance of the differences was assessed using the paired two-tailed Student's t test; ***, $p < 0.001$.

conceptual framework proposed by the latter suggests that relative efficacy results from the distinct preferences of the compounds for the active and inactive receptor conformers (Black and Shankley, 1995; Leff, 1995; Milligan et al., 1995). Thus our results suggest that it is by stabilizing R* that the overexpressed G proteins allosterically promote a reduction in inverse agonist binding. This hypothesis is further supported by the fact that the inverse agonist also showed reduced binding to the CAMβ_2AR.

The effect of G protein overexpression on inverse agonist binding reflected the coupling selectivity of the β_2AR since Gαs and Gαi, which are both known signaling partners of this receptor (Gilman, 1987; Kobilka, 1992; Crespo et al., 1995; Bogoyevitch et al., 1996; Daaka et al., 1997; Yamamoto et al., 1997), but not Gαq, which cannot couple to β_2AR (Offermanns and Simon, 1995), promoted the reduction in inverse agonist binding. The hypothesis that the reduction in binding capacity resulted from the functional coupling of the β_2AR to its cognate G proteins is also supported by the effects of Gpp(NH)p. Indeed, the receptor-G protein uncoupling promoted by the addition of the nonhydrolysable nucleotide during the binding assay partially inhibited the effect of G protein overexpression on inverse agonist binding. Although similar effects were observed upon overexpression of Gαi and Gαs, no conclusion on the relative affinity of the receptor for the two G proteins can be drawn since no information is available on their relative level of expression. However, the observation that both PTX and CTX led to an increase of ^{125}I-cyanopindolol binding capacity in HEK293s cells expressing endogenous levels of G proteins suggests that coupling to both Gi and Gs can occur at physiological level of G protein expression. These result also indicate that the influences of G protein on receptor isomerization are not limited to the insect system but can also be seen in mammalian cells expressing normal levels of G proteins. These results are entirely consistent with the observed reduction in ^{125}I-cyanopindolol binding previously reported by Krumins et al. (1997) upon elevation of Gαs expression using a dexamethasone-inducible promoter in S49 cells.

Although the maximal loss of inverse agonist binding observed in Sf9 cells was found upon overexpression of the Gαsβ1γ2 complex, significant effect could also be observed when either αs or the β1γ2 dimer was expressed individually. This may indicate that interaction with each of the G protein components is sufficient to partially stabilize R*. Alternatively, association of the overexpressed mammalian subunits with their endogenous insect partners (Hepler et al., 1993;

Butkerait et al., 1995; Richardson and Robishaw, 1999) may be responsible for the effects.

The observation that Gαsβ1γ2 overexpression could further decrease the binding of inverse agonists to the CAMβ_2AR suggests that the mutations do not irreversibly activate the receptor but rather shift the equilibrium toward R* and that further stabilization of the active conformer can still be achieved upon allosteric interactions with its cognate G proteins. This is consistent with the previous observation that inverse agonists can inhibit the spontaneous activity of CAMβ_2AR (Samama et al., 1993; Milligan et al., 1997). Reduced binding capacity of CAMβ_2AR when compared with β_2AR was previously attributed to the greater instability of the constitutively active mutant (Samama et al., 1993; Gether et al., 1997). Although this aspect was not directly assessed in the present study, our data suggest that the reduced ability of R* to bind to inverse agonists may also contribute to the lower binding generally obtained with this construct.

The allosteric effect of G protein overexpression on the binding of inverse agonists can be considered as the mirror image of the loss of agonist binding observed in many studies upon addition of nucleotides that promote receptor-G protein uncoupling. However, in most cases, such uncoupling is accompanied by a reduction of the affinity and not a loss of binding sites for agonists (Kenakin, 1996). Such a change in affinity and not B_{max} is in fact what the allosteric models based on ternary complex formation predict since R*G has a high affinity while R has a low affinity for agonists. Nevertheless, in a few cases, the nucleotide-promoted uncoupling leads to an apparent lost in agonist binding capacity (Bouaboula et al., 1997; Ohtaki et al., 1998). This could be interpreted as an indication that the low affinity state of the receptor for the agonist is too low for any binding to be detected under the experimental conditions used. A similar reasoning can be applied for the apparent loss of inverse agonist binding capacity observed in the present study following overexpression of the G proteins. In this situation, the low affinity state of the inverse agonists for the R*G forms of the receptor would be undetectable. The fact that the apparent loss of binding sites persists throughout the binding assay and that the inverse agonist is not able to convert the entire population of receptor into the inverse agonist-bound R form suggests that the conversion of at least a subpopulation of the receptors is occurring at a very slow rate. This may indicate that something is preventing the free and rapid isomerization between R*G and R. The excess in G protein itself could be responsible for this phenomenon as it could lock some R into the R*G form. Given that G proteins have been shown to be in large excess of receptors in several cell types (Alousi et al., 1991; Post et al., 1995; Milligan, 1996), it may not be surprising that we observed the phenomenon not only upon overexpression of Gi and Gs in Sf9 cells but also in mammalian HEK293s cells expressing endogenous levels of G proteins.

Although this is the first study systematically assessing the effect of the G protein overexpression on the direct binding properties of a radioligand series with various inverse efficacies, changes in both affinities and apparent B_{max} linked to alterations in the coupling state of GPCRs have previously been reported for a few inverse agonists. For example, guanine nucleotides treatment of membranes derived from cells expressing the human cannabinoid receptor CB1 (Bouaboula et al., 1997), or the serotoninergic $5HT_{2C}$ receptor (Barker et al., 1994; Westphal and Sanders-Bush, 1994) was found to increase the affinity and the number of binding sites detected by the inverse agonists [^{3}H]SR141716A and [^{3}H]mesulergine, respectively. [^{3}H]Spiperone binding capacity to the $5HT_{1A}$ receptor was also found to be increased upon GTP treatment (Sundaram et al., 1993). Also, increase Gαs expression was associated with a reduction in ^{125}I-cyanopindolol binding capacity (Krumins et al., 1997). More recently, overexpression of Gαi1 and Gαo led to a reduction of the affinity of the inverse agonist methiothepin for the $5HT_{5A}$ receptor (Francken et al., 2000).

The good correlation between inverse efficacy and the loss of binding promoted by G protein overexpression observed in the present study clearly establishes that signaling efficacy and binding to specific states (R versus R*) of the receptor are intimately linked. These results also indicate that the ratio between R and R* is under the dual influence of ligand binding and G protein coupling. It is therefore the resultant of the interactions within the ternary complexes that will ultimately determine the affinity states, the proportion of receptor in each of the affinity states, and the signaling efficacy for a given ligand. Whether R and R* each represent a discrete conformation or rather collections of receptor states cannot be directly addressed here. However, the observation that in some studies (Westphal and Sanders-Bush, 1994; Bouaboula et al., 1997) guanine nucleotide can affect simultaneously the measured affinity and the apparent B_{max} for inverse agonists suggest that more than two conformations are involved.

Our findings should also prove to be of practical importance when using radioligand binding assays to determine receptor number in various pathophysiological conditions. If, as it is often the case, the radioligands used have inverse efficacy, the conditions that modify the R and R* ratio could be erroneously interpreted as changes in the total receptor number. This is particularly important when considering that many pathological conditions are accompanied by alteration in G protein levels and thus could influence the R/R* equilibrium. For example, increased Gαs and Gαi levels reported in various cardiovascular diseases could be a contributing factor to the decrease in βAR density often detected with radiolabeled inverse agonists in these conditions (Schotten et al., 2000). Although the extent to which the allosteric effect contributed to the observed results cannot be easily determined, the use of neutral antagonists (when available) rather than inverse agonists as tracers may prove to be better choices.

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References

Alousi AA, Jasper JR, Insel PA, and Motulsky HJ (1991) Stochiometry of receptor-Gs-adenylate cyclase interactions. *FASEB J* **5:**2300–2303.

Barker EL, Westphal RS, Schmidt D, and Sanders-Bush E (1994) Constitutively active 5-hydroxytryptamine2C receptors reveal novel inverse agonist activity of receptor ligands. *J Biol Chem* **269:**11687–11900.

Barr AJ, Brass LF, and Manning DR (1997) Reconstitution of receptors and GTP-binding regulatory proteins (G proteins) in Sf9 cells. *J Biol Chem* **272:**2223–2229.

Barr AJ and Manning DR (1997) Agonist-independent activation of Gz by the 5-hydroxytryptamine1A receptor co-expressed in *Spodoptera frugiperda* cells: dis-

tinguishing inverse agonists from neutral antagonists. *J Biol Chem* **272:**32979–32987.
Black JW and Shankley NP (1995) Inverse agonists exposed. *Nature (Lond)* **374:**214–215.
Bogoyevitch MA, Anderson MB, Gillespie J, Clerk A, Glennon PE, Fuller SJ, and Sugden PH (1996) Adrenergic receptor stimulation of the mitogen activated protein kinase cascade and cardiac hypertrophy. *Biochem J* **314:**115–121.
Bond RA and Bouvier M (1998) Inverse agonists and G-protein-coupled receptors, in *Receptor-Based Drug Design* (Lett P ed) p 363–377, Marcel Dekker, New York.
Bond RA, Leff P, Johnson TD, Milano CA, Rockman HA, Mcminn TR, Apparsundaram S, Hyek MF, Kenakin TP, Allen LF, and Lefkowitz RJ (1995) Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the β_2-adrenoceptor. *Nature (Lond)* **374:**272–276.
Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, Maffrand JP, Le Fur G and Casellas P (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen activated protein kinase activation stimulated by insulin or insulin-like growth factor 1: evidence for a new model of receptor/ligand interactions. *J Biol Chem* **272:**22330–22339.
Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.
Butkerait P, Zheng Y, Hallak H, Graham TE, Miller H, Burris K, Molinoff PB, and Manning DR (1995) Expression of the human 5-hydroxytryptamine1A receptor in Sf9 cells. *J Biol Chem* **270:**18691–18699.
Chidiac P, Hebert TE, Valiquette M, Dennis M, and Bouvier M (1994) Inverse agonist activity of β-adrenergic antagonists. *Mol Pharmacol* **45:**490–499.
Chidiac P, Nouet S, and Bouvier M (1996) Agonist-induced modulation of inverse agonist efficacy at the β_2-adrenergic receptor. *Mol Pharmacol* **50:**662–669.
Colquhoun D (1973) The relationship between classical and cooperative models for drug action, in *A Symposium on Drug Receptors* (Rang HP, ed) pp 149–182, University Park Press, Baltimore.
Cotecchia S, Exum S, Caron MG, and Lefkowitz RJ (1990) Regions of the α_1-adrenergic receptor involved in coupling to phosphatidylinositol hydrolysis and enhanced sensitivity of biological function. *Proc Natl Acad Sci USA* **87:**2896–2900.
Crespo P, Cachero TG, Xu N, and Gutkind JS (1995) Dual effect of β-adrenergic receptors on mitogen activated protein kinase. *J Biol Chem* **270:**25259–25265.
Daaka Y, Luttrell DK, and Lefkowitz RJ (1997) Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. *Nature (Lond)* **390:**88–91.
De Lean A, Stadel JM, and Lefkowitz RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. *J Biol Chem* **255:**7108–7117.
Francken BJ, Josson K, Lijnen P, Jurzak M, Luyten WH, and Leysen JE (2000) Human 5-hydroxytryptamine receptors activate coexpressed G(i) and G(o) proteins in *Spodoptera frugiperda* 9 cells. *Mol Pharmacol* **57:**1034–1044.
Gardella TJ, Luck MD, Jensen GS, Schipani E, Potts JTJ, and Juppner H (1996) Inverse agonism of amino-terminally truncated parathyroid hormone (PTH) and PTH-related peptide (PTHrP) analogs revealed with constitutively active mutant PTH/PTHrP receptors. *Endocrinology* **137:**3936–3941.
Gether U, Ballesteros JA, Seifert R, Sanders-Bush E, Weinstein H, and Kobilka BK (1997) Structural instability of a constitutively active G protein-coupled receptor: agonist-independent activation due to conformational flexibility. *J Biol Chem* **272**:2587–2590.
Gether U and Kobilka BK (1998) G protein-coupled receptors. II. Mechanism of agonist activation. *J Biol Chem* **273**:17979–17982.
Gilman AG (1987) G-proteins: transducers of receptor-generated signals. *Annu Rev Biochem* **56:**615–649.
Grunewald S, Haase W, Reilander H, and Michel H (1996) Glycosylation, palmitoylation, and localization of the human D_{2S} receptor in baculovirus-infected cells. *Biochemistry* **35:**15149–15161.
Hepler JR, Kozasa T, Smrcka AV, Simon MI, Rhee SG, Sternweis PC, and Gilman AG (1993) Purification from Sf9 cells and characterization of recombinant Gqα and G11α: activation of purified phospholipase C isozymes by Gα subunits. *J Biol Chem* **268:**14367–14375.
Jansson CC, Kukkonen JP, Nasman J, Huifang G, Wurster S, Virtanen R, Savola JM, Cockcroft V, and Akerman KEO (1998) Protean agonism at α_{2A}-adrenoceptors. *Mol Pharmacol* **53:**963–968.
Karlin A (1967) On the application of "a plausible model" of allosteric proteins to the receptor for acetylcholine. *J Theor Biol* **16:**306–320.
Kenakin T (1995) Agonist-receptor efficacy. I. Mechanisms of efficacy and receptor promiscuity. *Trends Pharmacol Sci* **16:**188–192.
Kenakin T (1996) The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol Rev* **48:**413–633.
Kjelsberg MA, Cotecchia S, Ostrowski J, Caron MG, and Lefkowitz RJ (1992) Constitutive activation of the α_{1B}-adrenergic receptor by all amino acid substitutions at a single site. *J Biol Chem* **267:**1430–1433.
Kobilka BK (1992) Adrenergic receptors as models for G protein-coupled receptors. *Annu Rev Neurosci* **15:**87–114.
Krumins AM, Lapeyre JN, Clark RB, and Barber R (1997) Evidence for the shuttle model for Gsα activation of adenylyl cyclase. *Biochem Pharmacol* **54:**43–59.
Labrecque J, Fargin A, Bouvier M, Chidiac P, and Dennis M (1995) Serotonergic antagonists differentially inhibit spontaneous activity and decrease ligand binding capacity of the rat $5HT_{2C}$ receptor in Sf9 cells. *Mol Pharmacol* **48:**150–159.
Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature (Lond)* **227:**680–686.
Lee TW, Cotecchia S, and Milligan G (1997) Up-regulation of the levels of expression and function of a constitutively active mutant of the hamster α_{1b}-adrenoceptor by ligands that act as inverse agonists. *Biochem J* **325:**733–739.
Leeb-Lundberg LMF, Mathis SA, and Herzig MCS (1994) Antagonists of bradykinin that stabilize a G-protein-uncoupled state of the B_2 receptor act as inverse agonists in rat myometrial cells. *J Biol Chem* **269:**25970–25973.
Leff P (1995) The two-state model of receptor activation. *Trends Pharmacol Sci* **16:**89–97.
Leff P and Scaramellini C (1998) Promiscuity, pre-coupling and instability. *Trends Pharmacol Sci* **19:**130–133.
Lefkowitz RJ, Cotecchia S, Samama P, and Costa T (1993) Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol Sci* **14:**303–307.
Milligan G (1996) The stochiometry of expression of protein components of the stimulatory adenylyl cyclase cascade and the regulation of information transfer. *Cell Signal* **8**:87–95.
Milligan G, Bond RA, and Lee M (1995) Inverse agonism: pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol Sci* **16:**10–13.
Milligan G, MacEwan DJ, Mercouris M, and Mullaney I (1997) Inverse agonism at adrenergic and opioid receptors: studies with wild-type and constitutively active mutant receptors. *Recept Channels* **5:**209–213.
Morello JP, Salahpour A, Laperriere A, Bernier V, Arthus MF, Lonergan M, Petaja-Repo U, Angers S, Morin D, Bichet DG, and Bouvier M (2000) Pharmacological chaperones promote cell surface expression and functional rescue of V_2-vasopressin receptor mutants. *J Clin Invest* **105:**887–895.
Mouillac B, Caron M, Bonin H, Dennis M, and Bouvier M (1992) Agonist-modulated palmitoylation of β_2-adrenergic receptor in Sf9 cells. *J Biol Chem* **267:**21733–21737.
Mullaney I, Carr IC, and Milligan G (1996) Analysis of inverse agonism at the delta opioid receptor after expression in rat 1 fibroblasts. *Biochem J* **315:**227–234.
Offermanns S and Simon MI (1995) Gα15 and Gα16 couple a wide variety of receptors to phospholipase C. *J Biol Chem* **270:**15175–15180.
Ohtaki T, Ogi K, Masuda Y, Mitsuoka K, Fujiyoshi Y, Kitada C, Sawada H, Onda H, and Fujino M (1998) Expression, purification, and reconstitution of receptor for pituitary adenylate cyclase-activating polypeptide: large-scale purification of a functionally active G protein-coupled receptor produced in Sf9 insect cells. *J Biol Chem* **273:**15464–15473.
Post SR, Hilal-Dandan R, Urasawa K, Brunton LL, and Insel PA (1995) Quantification of signalling components and amplification in the beta-adrenergic-receptor-adenylyl cyclase pathway in isolated adult rat ventricular myocytes. *Biochem J* **311:**75–80.
Ren Q, Kurose H, Lefkowitz RJ, and Cotecchia S (1993) Constitutively active mutants of the α_2-adrenergic receptor. *J Biol Chem* **268:**16483–16487.
Richardson M and Robishaw JD (1999) The α_{2A}-adrenergic receptor discriminates between Gi heterotrimers of different $\beta\gamma$ subunit composition in Sf9 insect cell membranes. *J Biol Chem* **274:**13525–13533.
Salomon Y, Londos C, and Rodbell M (1974) A highly sensitive adenylate cyclase assay. *Anal Biochem* **58:**541–548.
Samama P, Cotecchia S, Costa T, and Lefkowitz RJ (1993) A mutation-induced activated state of the β_2-adrenergic receptor. *J Biol Chem* **268:**4625–4636.
Schotten U, Filzmaier K, Borghardt B, Kulka S, Schoendube F, Schumacher C, and Hanrath P (2000) Changes of β-adrenergic signaling in compensated human cardiac hypertrophy depend on the underlying disease. *Am J Physiol Heart Circ Physiol* **278:**2076–2083.
Seifert R, Wenzel-Seifert K, Lee TW, Gether U, Sanders-Bush E, and Kobilka BK (1998) Different effects of Gsα splice variants on β_2-adrenoreceptor-mediated signaling: the β_2-adrenoreceptor coupled to the long splice variant of Gsα has properties of a constitutively active receptor. *J Biol Chem* **273:**5109–5116.
Smith MJ, Leurs R, Alewijnse AE, Blauw J, Van Nieuw Amerongen PG, Van De Vrede Y, Roovers E, and Timmerman H (1996) Inverse agonism of histamine H_2 antagonists accounts for upregulation of spontaneously active histamine H_2 receptors. *Proc Natl Acad Sci USA* **93:**6802–6807.
Sundaram H, Newman-Tancredi A, and Strange PG (1993) Characterization of recombinant human serotonin $5HT_{1A}$ receptors expressed in Chinese hamster ovary cells: [^{3}H]spiperone discriminates between the G-protein-coupled and -uncoupled forms. *Biochem Pharmacol* **45:**1003–1009.
Thron CD (1973) On the analysis of pharmacological experiments in terms of an allosteric receptor model. *Mol Pharmacol* **9:**1–9.
Weiss JM, Morgan PH, Lutz MW, and Kenakin TP (1996) The cubic ternary complex receptor-occupancy model. I. Model description. *J Theor Biol* **178:**151–167.
Westphal R and Sanders-Bush E (1994) Reciprocal binding properties of 5-hydroxytryptamine type 2C receptor agonists and inverse agonists. *Mol Pharmacol* **46:**937–942.
Yamamoto J, Nagao M, Kaziro Y, and Itoh H (1997) Activation of p38 mitogen-activated protein kinase by signaling through G protein-coupled receptors: involvement of Gi and Gq/11 subunits. *J Biol Chem* **272:**27771–27777.

Address correspondence to: Dr. Michel Bouvier, Department of Biochemistry, Universitu of Montreal, 2900 Edouard Montpetit, Rm. D-360, P.O. Box 6128, Succ. Center-ville-Montreal PQ, H3C 3J7 Canada. E-mail: bouvier@bcm.umontreal.ca