0026-895X/01/5903-532–542$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 59:532–542, 2001

Vol. 59, No. 3
352/883034
Printed in U.S.A.

Inverse Agonist Activity at the α_{2A}-Adrenergic Receptor

SUSAN M. WADE, KENG-LI LAN, DORIAN J. MOORE, and RICHARD R. NEUBIG

Departments of Pharmacology (S.M.W., K-L.L., D.J.M., R.R.N.) and Internal Medicine/Hypertension (R.R.N.), The University of Michigan, Ann Arbor, Michigan

Received June 12, 2000; accepted November 17, 2000

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Constitutive activation of G protein-coupled receptors (GPCRs) is now well recognized and many classical GPCR antagonists have been found to be inverse agonists. For the α_{2A}-adrenergic receptor (AR) we determine the relative inverse efficacies of a series of antagonists and utilize the extended ternary complex model to estimate the fraction of constitutively active mutant (CAM) receptors in the active state. Stable Chinese hamster ovary cell lines expressing the porcine α_{2A}-AR in its wild-type (WT) and constitutively activated (CAM-T373K) form were isolated. Activation of both G_i and G_s was enhanced for CAM receptors. cAMP production was suppressed in cells with the CAM α_{2A}-AR and this suppression was reversed by α_2-adrenergic antagonists with an order of inverse efficacy of rauwolscine > yohimbine > RX821002 > MK912, whereas phentolamine and idazoxan were essentially neutral antagonists. This striking difference in inverse efficacy between idazoxan and RX821002 may account for in vivo pharmacological differences between these two α_2-adrenergic antagonists. Agonist binding affinity to the non-G protein-coupled CAM receptor was 3- to 9-fold higher than to WT, whereas binding of the most efficacious inverse agonists, yohimbine and rauwolscine, was 1.7- and 2.1-fold weaker. Analysis of this difference by the extended ternary complex model indicates that approximately 50% of the CAM α_{2A}-AR is in the active (R^*) state although there is no detectable constitutive activity of the WT receptor in the absence of agonist.

Receptors coupled to G proteins (GPCRs) play a major role in signal transduction and are the targets of a large number of therapeutic drugs. An important development in the understanding of GPCRs was the recognition that they could couple to (Wreggett and De Lean, 1984; Neubig et al., 1988) and functionally activate (Costa and Herz, 1989) G proteins in the absence of an agonist. These initial observations were buttressed by the identification of mutant receptors that had substantial constitutive activity (Kjelsberg et al., 1992; Parma et al., 1993; Shenker et al., 1993). This led to the "Extended Ternary Complex (ETC) model" (Samama et al., 1993) and the cubic ternary complex model (Weiss et al., 1996). In these models, the receptor exists in an equilibrium between an inactive state R and an active state R^* in the absence of drug. This equilibrium, differing for each receptor, determines basal activity. For wild-type receptors, R predominates and there is minimal receptor activity in the absence of agonist. Binding of agonist stabilizes R^* causing G protein coupling and activation of cellular responses. High levels of receptor expression or constitutively active mutant receptors increase the concentration of R^* inducing a response in the absence of agonist. In some disease states such as familial male precocious puberty (luteinizing hormone receptor) (Shenker et al., 1993), hyperfunctioning thyroid adenoma (thyrotropin receptor) (Parma et al., 1993), and retinitis pigmentosa (rhodopsin) (Rao et al., 1994), naturally occurring point mutations that lead to constitutive activation of GPCRs have been implicated.

Constitutively active GPCRs suggested the concept of inverse agonists (Barker et al., 1994; Chidiac et al., 1994; Bond et al., 1995)—drugs that preferentially bind to R and inhibit basal receptor activity. In contrast, neutral antagonists bind equally well to R and R^*, have no effect on basal receptor activity, but block the effects of both agonists and inverse agonists. Indeed, many drugs classically thought of as antagonists were subsequently found to have inverse agonist activity (Chidiac et al., 1994). Inverse agonists are expected to have therapeutic utility in the setting of CAM receptors such as in familial male precocious puberty or thyroid adenomas but more recently, they have been found to have unique physiological or biochemical effects (Nagaraja et al., 1999) even in the setting of relatively low levels of expression of wild-type receptors (Berg et al., 1999). Thus the differentiation of inverse agonist and neutral antagonist activity is important to our understanding of drug mechanisms.

Although the general features of the ETC model are accepted,

Supported by National Institutes of Health HL46417. Production and flow cytometry screening of the mutant receptors received assistance from UMMAC, National Institutes of Health P60-AR20557.

ABBREVIATIONS: GPCR, G protein-coupled receptor; CAM, constitutively active mutant; ETC, extended ternary complex; WT, wild-type; AR, adrenergic receptor; 5-HT, 5-hydroxytrytamine; PTX, pertussis toxin; IBMX, isobutylmethylxanthine; HA, hemagglutinin; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; GppNHp, 5′-guanylyimidodiphosphate; NLLSQ, non-linear least squares regression.

there have not been clear quantitative tests of that model. For example, it isn't clear what fraction of receptors are in the R^* state for WT and CAM receptors. Also, inverse agonists should have a lower affinity for the R^* state of GPCRs but the data demonstrating this is not clear. Most studies have shown limited ($\leq$2-fold) effects on inverse agonist affinity for CAM mutant receptors. Here we examine quantitative questions about the ETC model in the context of the α_{2A}-adrenergic receptor.

α_2-Adrenergic receptors (AR) play important physiological roles in the central control of blood pressure, pain, and neuronal excitability and the α_{2A}-AR subtype is the major contributor to these responses (MacMillan et al., 1996; Hein et al., 1999). Furthermore, α_2-AR agonists like clonidine are effective antihypertensive therapies although there remains some controversy over the relative role of the α_{2A}-AR and the putative I_1 imidazoline receptor in these actions. Like opioid receptors, α_2-ARs are well known to exhibit a pronounced excitatory withdrawal syndrome upon rapid cessation of chronic agonist therapy (Neusy and Lowenstein, 1989). The classical antagonist of the α_2-AR, yohimbine, causes central excitation but it is not clear whether the central excitatory effects of yohimbine are: 1) manifestations of pure antagonist activity at the α_2-AR, 2) due to the reported inverse efficacy of yohimbine (Tian et al., 1994), or 3) due to effects on other receptors such as 5HT receptors (Convents et al., 1989). Currently, there is insufficient pharmacological characterization of these α_{2A} antagonists to define such mechanisms. The identification of α_{2A} adrenergic inverse agonists and neutral antagonists that are more selective than yohimbine would be desirable. Furthermore, recent literature is contradictory as to whether the classical α_2-AR antagonists are inverse agonists or neutral antagonists (Tian et al., 1994; Wurch et al., 1999).

Lefkowitz and colleagues (Ren et al., 1993) showed that mutation of Thr-373 in the α_{2A}-AR results in CAM activity leading to basal inhibition of adenylyl cyclase and increased receptor affinity for agonist. We use this CAM α_{2A}-AR to evaluate α_2-antagonists for inverse agonist activity. We wished to determine inverse efficacy of α_2-AR antagonists and to examine quantitative predictions of the ETC model. We found inverse agonist activity among both alkaloid and imidazoline α_2-antagonists and identified a unique difference between idazoxan and RX821002, which may have important pharmacological implications. Also, we obtain estimates of the fraction of the α_{2A}-T373K mutant receptor in the R^* state and set an upper bound for that fraction for the WT α_{2A}-AR.

Materials and Methods

Radiochemicals. [2-^{3}H]Adenine (21–25 Ci/mmol) was from Amersham Life Science (Arlington Heights, IL). [^{3}H]Yohimbine (74.5–78 Ci/mmol) was from DuPont-New England Nuclear (Wilmington, DE).

Chemicals. Opti-MEM, LipofectAMINE, and Geneticin (G-418) were obtained from Life Technologies (Gaithersburg, MD). Fluorescein-labeled anti-[HA]-antibody was from Boehringer Mannheim (Indianapolis, IN). Pertussis toxin (PTX) and cholera toxin were from List Biological Laboratories (Campbell, CA). Forskolin was from Calbiochem (La Jolla, CA). UK-14,304 and prazosin were from Pfizer (Sandwich, England). Clonidine was from Boehringer Ingelheim (Ingelheim, Germany), idazoxan from Reckitt & Colman (Hull, England), MK912 a gift of Dr. Staffan Uhlén, Uppsala University, phenoxybenzamine from Smith Kline & French Labs (Philadelphia, PA), phentolamine from Ciba-Geigy (Summit, NJ), propranolol from Ayerst Laboratories (New York, NY), rauwolscine from Roth (Germany), and RX821002 from Research Biochemicals, Inc. (Natick, MA). Isobutyl-1-methyl-xanthine (IBMX), adenosine 5′-triphosphate (ATP), adenosine 3′:5′-cyclic monophosphate (cAMP), 5′-guanylyimidodiphophate (GppNHp), epinephrine and yohimbine were from Sigma (St. Louis, MO).

Construction of Mutant α_{2A}-Adrenergic Receptor Plasmids. The pα_2Tag H/N construct was described previously (Wade et al., 1999). It includes an HA-epitope-tagged porcine α_{2A}-adrenergic receptor with unique silent *Hin*dIII and *Nhe*I restriction sites at Ala-359 and Lys-376. Mutagenic cassette ligation was used to introduce annealed 52-mer oligonucleotides containing the Thr-373 to lysine constitutively activating mutation into the *Hin*dIII/*Nhe*I digested pα_2Tag H/N vector. The mutation of the modified region in the product was confirmed by restriction enzyme digestion utilizing a silent diagnostic *Nru*I restriction site and confirmed by DNA sequencing.

Cell Culture and Transfection. CHO-K1 cells were maintained in Ham's F-12 medium with 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2. Stable selection for mutants was maintained by the addition of 0.4 mg/ml active G-418.

CHO-K1 cells were cotransfected with the cDNA for HA-epitope-tagged WT or CAM (T373K) α_{2A}-adrenergic receptor in pCMV4 along with the pSV2neo plasmid containing the neomycin resistance gene (kindly provided by Dr. Jun Sadoshima, University of Michigan). The ratio of receptor to pSV2neo DNA was 5 to 1. The DNA was added in Opti-MEM with 6 μl of LipofectAMINE reagent per μg of DNA for 24 h. Cells were returned to complete growth medium and 72 h after the start of transfection, G-418 was added. After 2 to 3 weeks in selection medium, G-418 resistant cells were labeled with a fluorescein-conjugated 12CA5 anti-HA monoclonal antibody and single receptor-positive cells sorted into 96-well plates on a Coulter Elite ESP cell sorter. Using this method, 100% of CAM clones (13/13) expressed high levels of receptor (5 to 80 pmol/mg of protein). One CAM (C16) and 3 WT (L1, L9, and Tag19) clones were selected for further study.

CHO-K1 Membranes. Membranes were prepared as previously described (Wade et al., 1999). The final membrane pellets were resuspended in TME buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.6), snap frozen, and stored at −80°C. Protein was determined by Bradford protein assay (Bradford, 1976).

Radioligand Binding Assays. [^{3}H]Yohimbine binding assays were performed in 96-well plates with 2 to 5 μg of protein per well in a final volume of 100 μl as previously described (Neubig et al., 1985). For competition binding measurements, membranes were incubated with the indicated drugs in TME buffer in the presence of 10 nM [^{3}H]yohimbine at room temperature for 30 min and filtered using a Brandel cell harvester. Nonspecific binding was defined by 10 μM yohimbine.

Whole Cell cAMP Accumulation. Whole cell cAMP accumulation was determined in 24-well plates as previously described (Wade et al., 1999). Briefly, cells were plated with 1 μCi/well [^{3}H]adenine for 18 to 20 h before assay and where indicated, 100 ng/ml pertussis toxin or 5 μg/ml cholera toxin was included in this preincubation. Cells were washed once with DMEM, then the assay was initiated by adding DMEM containing 1 mM IBMX, 30 μM forskolin, and the indicated drugs. Cells were incubated 30 min at 37°C, and the reaction was terminated by aspirating the incubation medium and quenching with 1 ml 5% TCA containing 1 mM ATP and 1 mM cAMP. Acid soluble nucleotides were separated on Dowex and alumina columns as described by Salomon et al. (1974). cAMP accumulation was normalized by dividing the [^{3}H]cAMP counts by the total [^{3}H]nucleotide counts (sum of ATP and ADP counts from the Dowex columns and cAMP counts from the alumina columns).

Data Analysis. All data are reported as mean ± S.E.M. Fitted curves were determined using an unweighted nonlinear least-squares method in GraphPad Prism version 3.00 for Windows

(GraphPad Software, San Diego, CA, www.graphpad.com). Statistical analysis of K_d ratios for WT and CAM receptors used the two-tailed, one sample t test in GraphPad Prism to compare the experimental ratio with a theoretical value of 1.0.

Results

Radioligand Binding and Receptor Conformation. It is well recognized that receptor conformation states can be monitored by radioligand binding. Thus we examined quantitatively both agonist and antagonist affinities for the WT and CAM α_{2A}-AR. Saturation binding experiments with the radiolabeled α_2-antagonist [^{3}H]yohimbine in membranes from the WT-Tag19 and CAM-C16 cell lines revealed similar levels of receptor expression with B_{max} values of 25 and 19 pmol/mg of protein, respectively (Table 1 and Fig. 1). Mock-transfected (Neo) cells displayed negligible specific binding (data not shown). To assess the conformational equilibria of the receptor independent of G protein coupling, we measured UK-14,304 competition for [^{3}H]yohimbine binding in the presence of GppNHp. As expected, the CAM receptor membranes had a much higher affinity for the α_2-AR agonist UK-14,304 than did the WT receptor-containing membranes (Table 1). Three pieces of evidence indicate that this enhanced affinity (13 nM versus 92 nM) reflects receptor conformations and not G protein coupling. First, the binding was conducted in the presence of GppNHp, which should uncouple RG complex. Second, two-site fits (not shown) of the competition studies even in the absence of GppNHp revealed only a small percentage of the receptors (<20%) in the high-affinity agonist binding component and F-tests did not show a statistical improvement by two-site fits over one-site fits ($p > 0.5$). Third, direct [^{3}H]UK-14,304 binding studies showed only 2 to 3 pmol/mg of binding sites versus ~20 pmol/mg of [^{3}H]yohimbine binding sites (not shown). Thus, due to the high receptor expression levels in these stable cell lines (i.e., greater than the amount of G protein), most of the receptor is uncoupled from G protein and the increased affinity of the CAM receptor for UK-14,304 is due to the intrinsic conformational properties of the receptor rather than to altered G protein coupling.

To understand conformational changes in the CAM receptor and their relation to mechanisms of inverse agonism, we wanted to determine whether antagonist affinities were also altered for the CAM receptor. Earlier studies had reported little or no difference in antagonist binding for the CAM versus WT receptor (Ren et al., 1993; Wurch et al., 1999). Since yohimbine had previously been shown to be an inverse agonist for G_i activation (Tian et al., 1994), we carefully determined its K_d value for WT and CAM receptor-expressing membranes to see if the change in receptor conformation expected with the activating mutation would alter the [^{3}H]yohimbine binding. In a total of nine[1] saturation experiments, we found that in every case the K_d for CAM membranes was higher than that in WT. Although there was some variation in the absolute K_d values from experiment to experiment, the K_d ratio was very consistent at 1.67 ± 0.17 (n = 9) and was significantly different from 1.0 ($p < 0.01$, two-tailed one-sample t test).

The binding of a series of other agonists and antagonists was examined by [^{3}H]yohimbine competition studies in the presence of GppNHp to assess the intrinsic binding properties of the WT and CAM receptor (i.e., independent of G protein coupling). Figure 2 shows the results for two agonists and two antagonists, and a summary for all ligands is presented in Table 2. Epinephrine shows the largest effect of the CAM phenotype with a 9-fold increase in affinity. In contrast, the partial agonist clonidine exhibited only a 3.3-fold increase, whereas UK-14,304, discussed above, was more like epinephrine. Thus, as previously reported (Wurch et al., 1999), the degree of affinity enhancement by the CAM mutation correlates with the efficacy of the agonists for activating G_i. The effects of the CAM mutation on antagonist binding are more subtle but are clearly present. As noted above, [^{3}H]yohimbine binding affinity was decreased 1.67-fold in a series of nine experiments. In the competition binding data the K_i difference for yohimbine was similar but slightly smaller, 1.5-fold. Competition studies showed a range of effects on antagonist binding from no change with idazoxan (Fig. 2D) to a 2.1-fold decrease in affinity for rauwolscine (Fig. 2C). IC_{50} values from the competition studies were converted to K_i values (Table 2) by the Cheng-Prusoff method (1973) using the directly measured [^{3}H]yohimbine binding affinities. Discussion of the functional data in Table 2 follows the presentation of the inverse efficacy results.

CAM Receptor Constitutively Inhibits cAMP Accumulation. To evaluate the functional activity of the CAM receptor and the effects of potential inverse agonists, whole cell cAMP measurements were done. Forskolin-stimulated [^{3}H]cAMP production in CAM cells was about 20 to 25% of that seen in either mock-transfected (Neo) cells or in cells expressing the WT receptor, $p < 0.01$ for CAM versus Neo (Fig. 3, top). The cAMP level in cells with the CAM receptor was similar to that observed with the WT receptor in the presence of the full agonist UK-14,304. Pretreatment with pertussis toxin decreased the cAMP production in our Neo cells by about 60%, and a similar effect was seen with WT receptor expressing cells. In contrast, cAMP actually increased 3-fold in the CAM cells upon PTX treatment, abolishing the differences among the three cell types (Fig. 3, bottom, open bars). This indicates that the low cAMP production from the constitutive activity of the mutant was mediated via agonist-independent activation of G_i and that WT receptor has minimal constitutive activity on its own,

TABLE 1

Binding parameters of WT and CAM stable lines

[^{3}H]Yohimbine binding to WT-Tag19 membranes was performed in 96-well plates for 30 min at room temperature. Data represent the mean ± S.E.M. of three (yohimbine saturation binding) or four (UK-14,304 competition binding) separate experiments performed in duplicate or triplicate. The K_d ratio differs from 1.0 with a $p < 0.01$ (*) or $p < 0.002$ (**).

	[^{3}H]Yohimbine Saturation Binding		UK-14,304 Competition	
			K_i	
	B_{max}	K_d	−GppNHp	+GppNHp
	pmol/mg protein	*nM*	*nM*	
WT	25 ± 4	5.5 ± 1.7	75 ± 13	92 ± 9
CAM	19 ± 3	8.3 ± 2.5	8.6 ± 1.0	13 ± 4
Ratio		1.54 ± 0.11*[a]	0.12**	0.14**

[a] The value reported here is for the three studies with Tag19 WT cell line. The value of 1.67 described in the text is from a larger series of experiments with additional WT cell lines.

[1] In addition to the three experiments with the WT-Tag 19 line (shown in Table 1), K_d values were also determined in paired experiments comparing CAM with WT-Tag L1 (n = 5) and WT-Tag L9 (n = 1).

despite the high-expression level (25 pmol/mg of protein). When the agonist UK-14,304 was added to WT receptor-containing cells in the absence of PTX, cAMP production was inhibited, however cAMP was stimulated by UK-14,304 in pertussis-treated WT cells. This is consistent with previous reports of dual coupling of α_{2A}-AR to both G_i and G_s (Eason et al., 1992; Wade et al., 1999). In CAM cells, however, UK-14,304 stimulated cAMP production both with and without pertussis pretreatment (Fig. 3). This suggests that the G_i response is already fully saturated in CAM cells before the agonist is added so only a G_s response is observed. UK-14,304 had no effect on adenylyl cyclase activity in mock-transfected (Neo) cells (Brink et al., 2000).

To further define the mechanisms of the complex effects of agonists on cAMP in the CAM and WT cells, epinephrine dose-response curves and effects of pertussis and cholera toxins were examined. Epinephrine showed a biphasic effect on cAMP production in CHO cells expressing the WT α_{2A}-AR; inhibition at 1 to 10 nM and a superimposed increase from 100 to 1000 nM (Fig. 4, top). The inhibition of adenylyl cyclase activity was abolished by pretreatment of cells with pertussis toxin revealing a pure stimulation (Fig. 4, top panel). This was very similar to results previously reported with UK-14,304 in CHO cells (Eason et al., 1992; Wade et al., 1999). The effects are mediated by the α_{2A}-AR and not an endogenous β-AR as there was minimal effect on cAMP production in Neo cells, and the effects were blocked by yohimbine but not propranolol (Brink et al., 2000). Similar results were found in the same cell line with UK-14,304 (Wade et al., 1999).

Epinephrine dose-response curves in cells expressing the CAM receptor also indicate enhanced G_s activation by the CAM α_{2A}-AR. Epinephrine caused an increase in forskolin-stimulated cAMP accumulation both with and without pertussis treatment (Fig. 4, bottom) apparently increasing adenylyl cyclase activity via G_s. This was confirmed by cholera toxin pretreatment after which there was no receptor-stimulated change in cAMP accumulation with epinephrine. The potency for G_s activation in CAM cells was significantly greater than in WT cells with EC_{50} values of 3.8 and 210 nM,

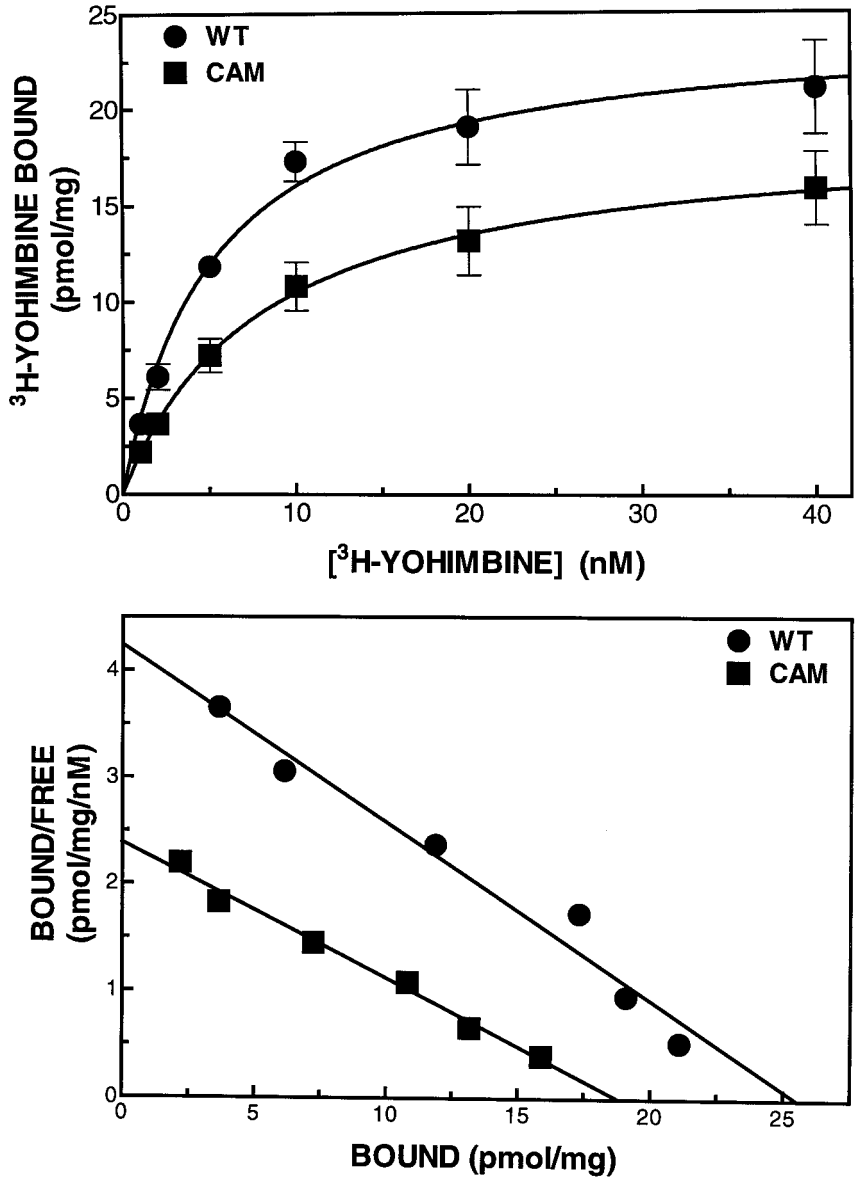

Fig. 1. Yohimbine saturation binding of WT and CAM porcine α_{2A}-AR. Saturation binding to WT-Tag19 (●) or CAM (■) membranes was performed in 96-well plates using 1 to 40 nM [^{3}H]yohimbine for 30 min at room temperature. Nonspecific binding, defined in the presence of 10 μM yohimbine, represented less than 10% of the total binding and was subtracted. Data represent the mean ± S.E.M. of three separate experiments performed in duplicate. The Scatchard plot of the saturation binding data is shown in the bottom panel.

respectively, in the presence of pertussis pretreatment. The 50-fold increase in potency is greater than the observed difference in epinephrine binding affinity for CAM and WT receptors (9-fold) suggesting that the CAM receptor is more efficiently activating G_s as well as G_i (see also evidence for constitutive activation of G_s described below).

Inverse Efficacies of α_2-Adrenergic Antagonists. We next looked at the effects of antagonists on forskolin-stimulated cAMP accumulation in CAM cells to determine whether inverse agonist activity would be observed. Rauwolscine was reported by Tian et al. (1994) to have the greatest inverse agonist activity at [^{35}S]GTPγS binding in PC12 cells. Consistent with that, we saw a striking reversal of the CAM receptor-suppressed cAMP production by that drug. Cholera toxin pretreatment of CAM cells did not affect the rauwolscine-mediated increase in cAMP accumulation (data not shown), but the increase was completely abolished by PTX pretreatment (Fig. 5), evidence of the G_i dependence of this response. Indeed there is a small decrease in cAMP with rauwolscine in PTX-treated CAM cells suggesting a small degree of constitutive activation of G_s. The WT receptor showed strikingly different results. In contrast to the constitutive activity reported for the WT rodent α_{2A}-AR in PC12 cells (Tian et al., 1994), we saw no increase in cAMP with rauwolscine with the WT porcine α_{2A}-AR in our CHO cells (Fig. 5, open squares). Thus despite very high levels of receptor expression (25 pmol/mg of protein) there was no evidence with either rauwolscine (Fig. 5) or PTX (Fig. 3) for constitutive activity of the WT receptor. Four of the five competitive antagonists tested showed inverse agonist activity in which the CAM receptor-suppressed cAMP production was significantly increased (Fig. 5, bottom). The sole neutral antagonist was idazoxan. That these were inverse agonist effects and not agonist effects is evident since the increase in cAMP was mediated by a PTX-sensitive G_i-dependent mechanism like that of rauwolscine rather than a G_s-dependent mechanism like epinephrine or UK-14,304 (Fig. 5, bottom).

Finally, we wanted to further characterize the inverse efficacy of a series of α_2-antagonists. Drugs were assayed for their ability to stimulate cAMP production in forskolin-stimulated CAM cells over a range of concentrations (Fig. 6 and Table 2). Antagonists examined for their inverse agonist activity in whole cell adenylyl cyclase assays displayed a rank order of efficacy of rauwolscine > yohimbine > RX821002 > MK912, whereas phentolamine and idazoxan had little or no effect on cAMP production. Phenoxybenzamine, an irreversible α-AR blocking agent, the α_1-antagonist prazosin and propranolol, a β-AR antagonist, also had no effect on cAMP accumulation (Table 2).

A comparison of the binding affinities of the antagonists

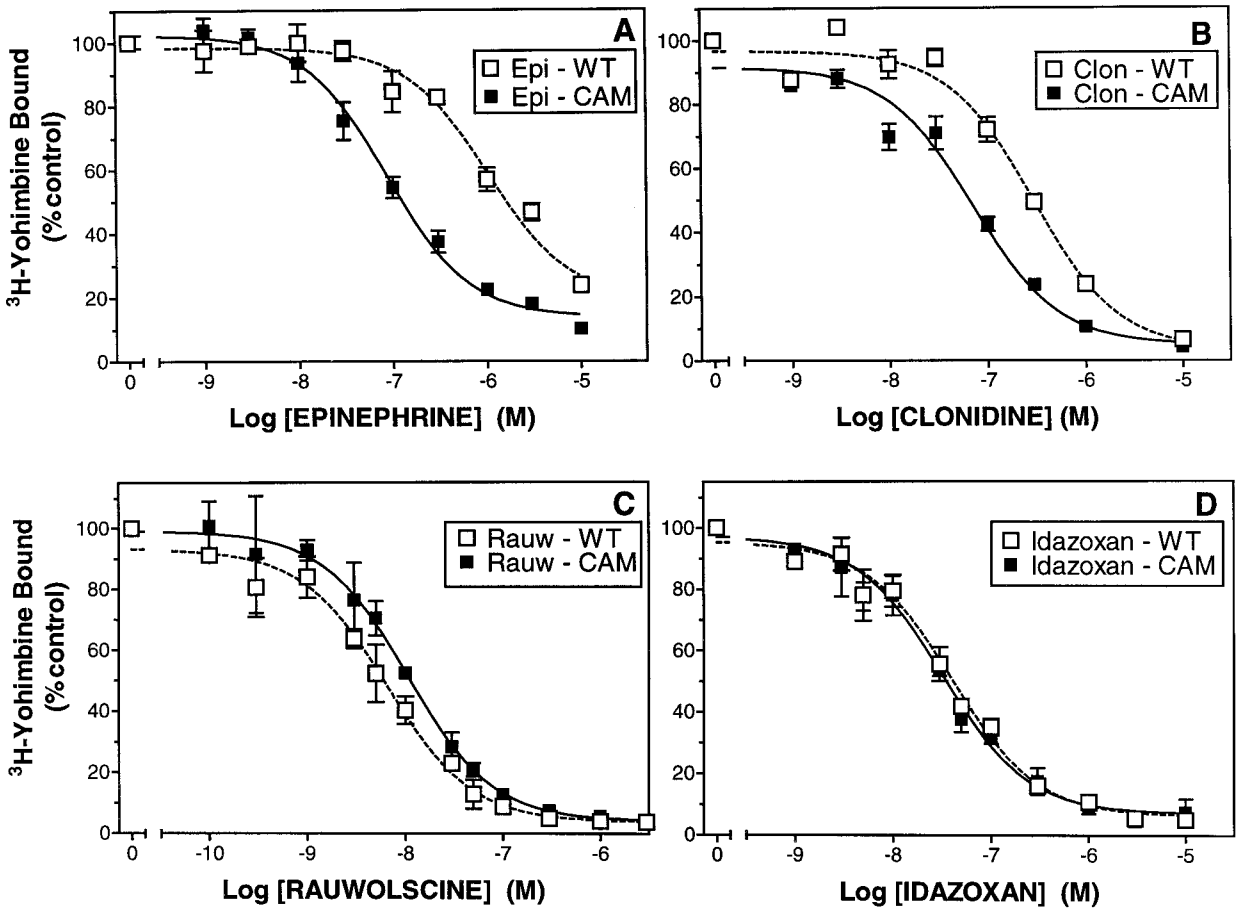

Fig. 2. Binding of selected agonists and antagonists at the WT and CAM α_{2A}-AR. Competition binding for 10 nM [^{3}H]yohimbine in the presence of 10 μM GppNHp was performed for 30 min at room temperature with WT-L1 or WT-L9 (□) and CAM (■) membranes. The agonists epinephrine and clonidine are shown in panels A and B, respectively, whereas panels C and D depict the antagonists rauwolscine and idazoxan. Data represent the mean ± S.E.M. of two (rauwolscine), three (epinephrine and clonidine), or four to five (idazoxan) separate experiments performed in duplicate. The rauwolscine data represents two experiments using the WT-L1 membranes; five additional experiments using the WT-L9 membranes gave similar results.

with their inverse efficacy is revealing (Table 2). The K_i values in this table are derived from NLLSQ fits of averaged curves from the indicated number of competition experiments. Rauwolscine showed the largest affinity difference and had the greatest inverse efficacy, whereas idazoxan showed no difference in affinity between CAM and WT receptors and was a neutral antagonist. The other four inverse agonists, yohimbine, RX821002, MK912, and phentolamine, had intermediate affinity differences. Because of the small magnitude of the changes in binding affinity, extra replicates of the rauwolscine and idazoxan experiments were done with more closely spaced concentrations (three per decade) and the curves (seven for rauwolscine and five for idazoxan) were analyzed individually for statistical treatment. As with the fits of the pooled data, rauwolscine showed a significant increase in K_i in each individual experiment. The mean ratio of K_i for CAM/WT was 2.24 ± 0.28, which was significantly different from 1.0 ($p < 0.005$, two-tailed one-sample t test) whereas idazoxan had a ratio of 0.99 ± 0.15, which was not significantly different from 1.0 ($p > 0.95$). A theoretical analysis of the expected affinity differences due to conformational changes in the extended ternary complex model is presented in the *Appendix*. The magnitude of the K_i ratio and the degree of cAMP increase indicates that approximately 50% of the CAM receptor is in the R^* state before agonist binding (see full analysis and assumptions under *Appendix*).

A final point about the inverse agonists to note in Table 2 is the difference between the K_i values for binding competition and the EC_{50} values for the increase in cAMP. In each case, the EC_{50} is greater than the K_i (5- to 50-fold). This is a somewhat unusual pharmacological behavior in that spare receptors will lead to a "left-shift" of the dose-response curve compared with the binding but in this case we have a "right-shift". Some of the difference may be due to different conditions of the two assays—Tris buffer with no sodium versus DMEM, which has sodium chloride—but this seems unlikely as sodium chloride tends to increase the affinity of antagonists at the α_{2A}-AR (Limbird et al., 1982). Thus the results seem more consistent with a "threshold" behavior in which no response is seen until a significant portion of the receptors is occupied. This could occur if there were sufficient receptors activated by the CAM mutation to produce a "spare receptor" phenomenon for adenylyl cyclase inhibition. In this case, occupancy of 50% of the receptors by inverse agonist would not be sufficient to reverse the spontaneous inhibition by 50%. An even greater degree of receptor occupancy (say 80–90%) might be required. In this case, the EC_{50} would be significantly greater than the measured K_d. This type of mechanism would not be surprising in the context of high-receptor expression, and a large number of spare receptors previously demonstrated for the α_{2a}-AR agonist response at the WT receptor (Brink et al., 2000).

Discussion

We have used a constitutively active mutant of the α_{2A}-AR stably expressed in CHO-K1 cells to examine mechanisms of α_{2A}-AR signaling. From these studies, at least three new conclusions can be drawn. First, the α_{2A}-AR T373K has enhanced signaling to both G_i and G_s. Second, the inverse efficacy of a series of α_2-AR antagonists is examined, and a novel difference between idazoxan and methyl-idazoxan (RX821002) has been found. This point is important for interpreting physiological studies that use these two compounds. Finally, the fraction of active α_{2A}-AR for the T373K CAM mutant is approximately 50% for our whole cell cAMP accumulation assay, whereas the WT receptor appears to have a very low percentage of receptor active in the absence of agonist.

The effects of constitutively active receptor mutants on different G proteins are not always concordant as reported for the α_1-AR by Perez et al. (1996). Ren et al. (1993) previously showed for the α_{2A}-AR that the T373K mutation adjacent to TM6 causes dramatically enhanced basal activation of

TABLE 2

Table of binding affinities and relative inverse agonist efficacies of drugs

K_i values were calculated from IC_{50} values determined by competition binding for 10 nM [^{3}H]yohimbine in the presence of 10 μM GppNHp (Fig. 2). Membranes were incubated for 30 min at room temperature in the presence of increasing concentrations of the indicated drugs. Relative affinities are the CAM K_i divided by the WT K_i for each drug. Data are from two to seven separate experiments performed in duplicate. EC_{50} values and maximum [^{3}H]cAMP produced were determined from dose-response curves for the indicated drugs using whole cell adenylyl cyclase assays (Fig. 6). CAM cells were incubated for 30 min at 37°C in the presence of 1 mM IBMX, 30 μM forskolin, and increasing concentrations of the indicated drugs. Data are from three to seven separate experiments performed in duplicate except for phenoxybenzamine and prazosin, which are single experiments.

Drug	[^{3}H]Yohimbine Binding			EC_{50}	[^{3}H]cAMP
	K_i		Relative Affinity CAM/WT (n)		Max[^{3}H]cAMP Produced
	WT	CAM			
	nM			*nM*	*% control*
Epinephrine	460	49	0.11 (3)	7.7	370
UK-14,304	55	7.6	0.14 (3)	7.1	660
Clonidine	130	40	0.30 (3)	N.D.	N.D.
Rauwolscine	5.3	11.2	2.1 (7)	52	720
Yohimbine	5.6	8.3	1.5 (5)	81	640
RX821002	2.1	3.1	1.5 (5)	160	480
MK912	2.2	3.8	1.7 (4)	87	360
Phentolamine	5.9	8.2	1.4 (3)	1700	210
Idazoxan	18.0	18.3	1.0 (5)	—[a]	≈100
Phenoxybenzamine	>200	>500	N.D. (2)	—[b]	≈100
Prazosin	>10,000	>10,000	N.D. (2)	—[b]	≈100
Propranolol	>10,000	>10,000	N.D. (2)	—[c]	≈100

N.D., not determined.

[a] No increase over control up to 1 mM ($n = 2$).

[b] No increase over control up to 1 μM ($n = 1$).

[c] No increase over control up to 100 μM ($n = 2$).

G_i, a result that we confirm here. We also demonstrate enhanced activation of G_s by the T373K mutant. This appeared as a modest degree of basal G_s response with the T373K mutant (Fig. 5, filled circles), but a more pronounced effect was seen on the EC_{50} for epinephrine-induced G_s activation. The EC_{50} was reduced >50-fold for the mutant versus WT whereas the K_d was reduced less than 10-fold, indicating enhanced G_s coupling. This does not appear to be just a shift in activity for G_i versus G_s since G_i effects were eliminated in this experiment by PTX treatment. However, this is difficult to evaluate quantitatively in part due to the fact that the α_{2A}-AR has a much reduced ability to activate G_s compared with G_i (Eason et al., 1992; Brink et al., 2000). Wurch et al. (1999) found increased G_z coupling by the T373K mutant in a transient cotransfection system with mouse $G\alpha_{15}$ in COS-7 cells using inositol phosphate production as their readout. Thus, the T373K mutant of the α_{2A}-AR appears to have a generalized ability to activate both G_i and G_s although quantitatively a comparison of the degree of activation is not possible.

Interestingly, the pharmacological behavior of this basal G_z activation was distinct from our results. For adenylyl cyclase inhibition, we show a clear order of inverse efficacy of rauwolscine > yohimbine > RX821002 > MK912 with RX821002 having substantial inverse efficacy. Phentolamine and idazoxan were neutral antagonists. In contrast, Wurch et al. (1999) reported that the basal G_z-mediated PLC activation with the T373K α_{2A}-AR could not be attenuated by RX821002, MK912 or idazoxan. They did not, however, report results with the most efficacious inverse agonists, rauwolscine or yohimbine. We would have expected a significant reduction in activation of G_z by RX821002 if it behaved in a manner similar to what we observe for G_i. Studying the rat α_{2D}-receptor (which is an ortholog of the human and porcine α_{2A}-AR), Tian et al. (1994) used [^{35}S]GTPγS binding in PC12 cells to show that yohimbine, rauwolscine, phentolamine, and idazoxan were all inverse agonists. They reduced basal GTPγS binding by 30 to 40% but that group did not test RX821002 or MK912. Interestingly, their ability to detect constitutive signaling (and inverse agonist effects) was abolished when sodium chloride was included in the assay.

For the endogenous α_{2C}-receptor in HepG2 cells, Cayla et al. (1999) found that treatment with RX821002 or yohimbine resulted in a significant increase in receptor number but phentolamine did not. They concluded that RX821002 and

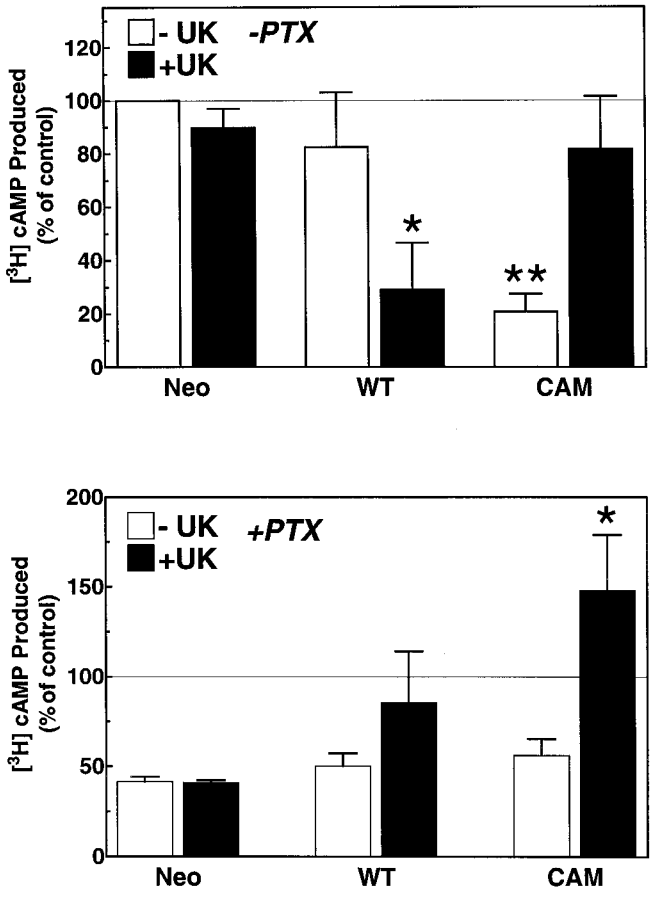

Fig. 3. Pertussis-sensitivity of transfected cell lines in the presence or absence of agonist. Whole cell adenylyl cyclase assays were performed on mock-transfected (Neo), WT, and CAM cells with (bottom panel) or without (top panel) PTX pretreatment. Cells were incubated with (filled bars) or without (open bars) 10 nM UK-14,304 for 30 min at 37°C in the presence of 1 mM IBMX and 30 μM forskolin. Results are the mean ± S.E.M. of three separate experiments performed in duplicate. Forskolin-stimulated [^{3}H]cAMP production in CAM cells was about 20 to 25% of that seen in Neo cells. Values differing significantly from control (Neo without UK-14,304) are marked, $p < 0.05$ (*), $p < 0.01$ (**).

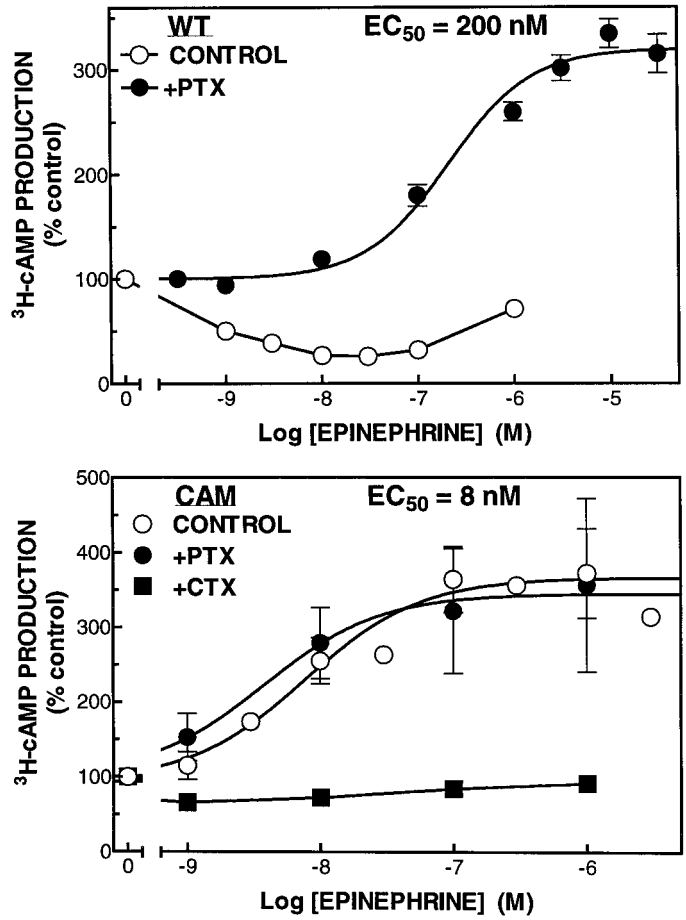

Fig. 4. Agonist-mediated cyclase activity in WT and CAM cells. Whole cell adenylyl cyclase assays were performed on WT (top panel) or CAM (bottom panel) cells that had been pretreated with (●) or without (○) PTX or with cholera toxin (■). Cells were incubated with increasing concentrations of epinephrine for 30 min at 37°C in the presence of 1 mM IBMX and 30 μM forskolin. Data are the mean ± S.E.M. of two, three (WT + PTX), or four (CAM control) separate experiments performed in duplicate or triplicate.

yohimbine thus behaved as inverse agonists, whereas phentolamine behaved as a neutral antagonist in this system. Although this was a different receptor subtype and a different experimental readout, their pharmacological profile completely agrees with ours. Interestingly, we were not able to demonstrate any significant changes in α_{2A}-receptor number after treatment of either WT or CAM α_{2A}-AR with UK-14,304 or rauwolscine (data not shown). This difference may be due to the well known resistance of α_{2A}-AR to internalization and the substantial pool of α_{2C}-AR, which is intracellular in the absence of agonist (Daunt et al., 1997).

Thus several factors, such as the type of assay, receptor subtypes, cell type, and local cellular G protein concentrations may affect constitutive receptor activity and thus will be important for detection of inverse agonist activity at a receptor subtype (for review, see Pauwels and Wurch, 1998). Interestingly, the best concordance of the pharmacological data mentioned above is between our results with α_{2A}-AR-mediated inhibition of cAMP accumulation and the receptor up-regulation studies of Cayla et al. (1999) with α_{2C}-AR. Yohimbine and RX821002 were inverse agonists in both studies. There is a direct contradiction between our results and those of Tian et al. (1994) in that phentolamine and idazoxan are clearly neutral antagonists in our hands. In particular, idazoxan showed both neutral antagonist behavior in the functional assay and absolutely no preference for R^* in the binding studies.

One of the other major questions that we address here is the fraction of receptor that is active in the absence of agonist. Also, we wanted to understand why CAM mutations have minimal effect on apparent affinity of even the most efficacious inverse agonists. The two-state ETC model has been frequently used to explain constitutive activation and inverse agonists (Samama et al., 1993), and it would predict that an inverse agonist must have a substantial difference in affinities for R and R^*. Although some recent data indicate that either an induced fit model or multiple active receptor states may be required to account for observations such as agonist-directed trafficking of receptor stimulus (Kenakin, 1995) or differential constitutive activation of signaling pathways (Perez et al., 1996), the ETC model is still a useful starting point for analysis. Although a lower affinity of inverse agonists for CAM receptors has been observed (Costa and Herz, 1989; Samama et al., 1994), the effects were very small (2-fold), and numerous other studies have not reported such an effect (Kjelsberg et al., 1992; Ren et al., 1993). In our theoretical examination of this question (see *Appendix*), we show that the expected changes in antagonist affinity are indeed modest and depend on the degree to which the receptor is spontaneously in the active state (i.e., depends on L, the equilibrium constant governing the R to R^* equilibrium). Only if a very large fraction of the receptor (i.e., >90%) is spontaneously in the R^* state would you expect to see a *large* affinity shift of an inverse agonist for a WT versus CAM receptor. The approximately 2-fold shift that we see for rauwolscine is consistent with approximately half of the CAM receptor being in the R^* state (see *Appendix* for details). Furthermore, the lack of inverse agonist effect with the WT α_{2A}-AR despite very high expression indicates that a very small percentage of the WT receptor is active in the absence of agonist. This is in contrast to the β_2-AR for which the WT receptor appears to have approximately 7% of the activity of the CAM mutant (Samama et al., 1993).

This very low level of constitutive activity of the WT α_{2A}-AR seems in conflict with results of Tian et al. (1994). They used PC-12 cells stably expressing a cloned rat α_{2D}-AR to look at the effects of rauwolscine on [^{35}S]GTPγS binding to

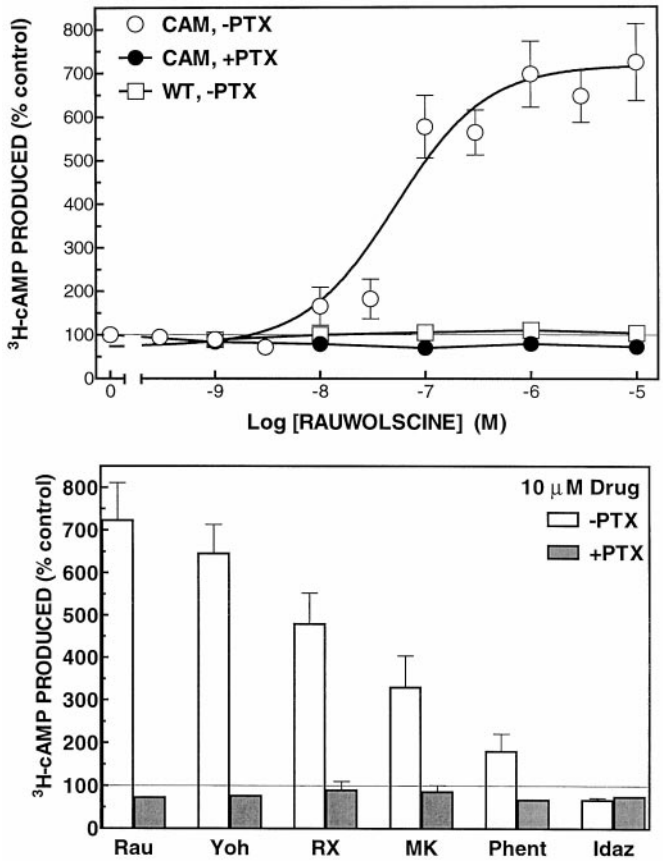

Fig. 5. Pertussis-sensitivity of inverse agonists in CAM cells. Whole cell adenylyl cyclase assays were performed on cells for 30 min at 37°C in the presence of 1 mM IBMX and 30 μM forskolin. Top panel, CAM cells pretreated with (●) or without (○) PTX and untreated WT cells (□) were incubated with increasing concentrations of rauwolscine. CAM data are the mean ± S.E.M. of two (PTX treated) or seven (control) separate experiments; WT data are from three separate experiments. Bottom panel, CAM cells that had been pretreated with (filled bars) or without (open bars) PTX were incubated with 10 μM of the indicated drugs. Data are the mean ± S.E.M. of three to six separate experiments for untreated cells and one or two separate experiments for PTX-treated cells. All experiments were performed in duplicate.

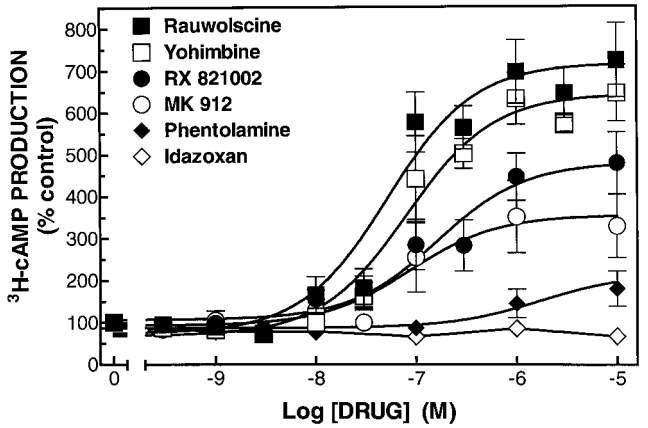

Fig. 6. Range of inverse efficacy of α_2 antagonists. Whole cell adenylyl cyclase assays on CAM cells were carried out for 30 min at 37°C in the presence of 1 mM IBMX, 30 μM forskolin, and increasing concentrations of the indicated drugs. Data are the mean ± S.E.M. of three to seven separate experiments performed in duplicate.

membrane preparations. The α_{2D}-AR is the rodent ortholog of the human or porcine α_{2A}-AR. They found that rauwolscine was able to decrease basal [^{35}S]GTPγS binding and that this decrease was dependent on both the level of receptor expression and upon sodium concentration in their assay (as noted above). Furthermore, their pharmacological profile of inverse efficacy was very different from ours. There are many potential explanations for the differences between our results and theirs: 1) the different species of origin of the receptor (rat versus pig), 2) different cell types used (CHO versus PC12), 3) the different readout of the assay (GTPγS binding versus adenylyl cyclase inhibition), and 4) the presence of sodium in our intact cell cAMP accumulation assays, which was absent from their nucleotide binding studies. Sodium, which is present in intact systems, has been shown to suppress basal activity for opioid receptors (Costa et al., 1990).

A final observation relates to different inverse efficacies of closely related imidazoline compounds. A role has been postulated for a novel imidazoline I_1 type receptor in mediating many effects of clonidine and other imidazoline-containing α_2 agonists (Ernsberger and Haxhiu, 1997). One pharmacological distinction between imidazoline receptor effects and those of α_2-AR has been the difference between effects of idazoxan and its methyl-substituted congener RX821002. This difference has been ascribed to selectivity of RX821002 to bind to the α_2-AR but not to the putative I_1 receptor. In contrast, idazoxan binds to both types of sites (Meana et al., 1997). Interestingly in our study, RX821002 (2-methoxy idazoxan) is a fairly efficacious inverse agonist whereas idazoxan is a neutral antagonist. Although different types of evidence have pointed to the I_1 receptor as the mediator of imidazoline drug-induced physiological effects, differences between the actions of RX821002 and idazoxan or other compounds must now take into account the relative inverse efficacy of these drugs at the α_{2A}-AR.

In conclusion, we describe the relative inverse efficacies of a series of α_{2A}-AR antagonists and the relation of inverse efficacies to binding properties. We provide new information both about α_{2A}-adrenergic receptor conformational regulation as well as potentially important pharmacological distinctions among different α_{2A}-AR antagonists.

Appendix

Estimation of the Fraction of CAM and WT Receptor in the Active Conformation

Although it is clear that constitutively activated receptors have a greater fraction of receptor in the active state in the absence of agonist than do wild-type receptors, in most cases it has not been determined what fraction of receptors are active in the CAM receptor. The extended ternary complex model describes the relation between ligand affinity for the two states of a receptor and the conformational changes of the receptor, which occur upon ligand binding. In the simplest version of this model[2] (Fig. 7), there are two receptor states: inactive, R, and active, R^*. Since the affinity of an

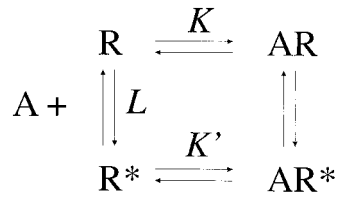

Fig. 7. Extended ternary complex model. Simple version of the extended ternary complex model in which the receptor exists in two states, an inactive R and an active R^* state. K and K' represent the affinities of the ligand (A) for the inactive and active states, respectively, whereas L represents the initial equilibrium between the inactive and active conformations of the receptor.

inverse agonist is lower for R^* than for R, we may be able to use changes in *inverse agonist* binding to estimate the fraction of a CAM receptor in the R^* state. This analysis is based on the assumption that the mechanism of constitutive activation is a change in the equilibrium between the R and R^* states without a significant change in the microscopic equilibrium constant for ligand binding to the basal receptor state R (K). In addition to the changes in antagonist binding, *agonist* binding affinities may also provide information about the fraction of active receptor. However, the agonist binding analysis may be complicated by the profound effects of G protein coupling on agonist binding and by the fact that agonist itself will increase the fraction of R^*.

What is the expected binding of an inverse agonist to a CAM receptor compared with its binding to a WT receptor? There are two parameters that determine both ligand binding and the amount of active receptor R^* in the presence of ligand (i.e., the initial equilibrium between the active and inactive conformations, $L = R^*/R$, and the ratio of affinities of the ligand for the active and inactive state, K'/K). Two assumptions that we make for this analysis are: 1) that the only mechanism by which the inverse agonist effects a change in receptor activity is by its differential affinity (K'/K) for the active (R^*) and inactive (R) states of the receptor, and 2) that the only difference between the WT and CAM receptor is a different value of L, the parameter describing the equilibrium between the active and inactive state in the absence of ligand.

One might think that if there were appreciable R^* present for the CAM receptor, that one should simply see a biphasic binding curve for the inverse agonist in which the low-affinity population would represent R^*. As derived below [and described previously (Neubig et al., 1985)], an equilibrium model such as in Fig. 7 predicts a simple hyperbolic binding function despite the presence of two states. However, the apparent K_d is a weighted average of the three parameters, L, K, and K'. Thus we must look at the predicted *affinity shifts* from a WT to a CAM receptor rather than just at the shape of the binding curve for the CAM receptor. The equations[3] describing the behavior of this system for binding of the antagonist A are as follows:

$$R_T = R + R^* + AR + AR^*$$

$$R^* = L \cdot R$$

$$AR = K \cdot A \cdot R$$

$$AR^* = L \cdot K' \cdot A \cdot R$$

[2] We have left out the G protein component for the calculations involving inverse agonist binding because the receptor density (~20 pmol/mg of protein) is greater than that of G_i (~10 pmol/mg for CHO cells) (Gerhardt, 1992), and we have included GppNHp in the binding assays to further reduce any G protein contribution. Chen et al. (2000) recently compared the ETC and cubic ternary complex models but did not address the question of inverse agonist affinities for the different receptor states.

[3] In these calculations, we use association equilibrium constants to simplify the calculations. The conclusions would be the same if dissociation constants were used.

The apparent affinity of a ligand for this binding system will depend on all three of the parameters and can be calculated as follows.

The binding occupancy function is

$$\text{Fraction bound} = \frac{AR + AR^*}{R + R^* + AR + AR^*}$$

$$= \frac{K \cdot A + L \cdot K' \cdot A}{1 + L + K \cdot A + L \cdot K' \cdot A}$$

which can be rearranged to

$$\frac{AR + AR^*}{R + R^* + AR + AR^*} = \frac{(K + L \cdot K') \cdot A}{1 + L + (K + L \cdot K') \cdot A}$$

or

$$\frac{AR + AR^*}{R + R^* + AR + AR^*} = \frac{A}{(1 + L)/(K + L \cdot K') + A}$$

This is the equation of a hyperbolic binding function with an apparent K_d of

$$K_d = (1 + L)/(K + L \cdot K') \quad (1)$$

The simplest formulation of the extended ternary complex model to account for constitutive receptor activation assumes that the only change to the receptor is the equilibrium constant (L) for transitions between R and R^* changes whereas the relative binding affinities (K and K') of agonist for R and R^* are not altered by a CAM mutation.[4] So for an inverse agonist (or actually for any drug), the ratio of the binding affinities for WT and CAM receptors would be

$$K_d(\text{CAM})/K_d(WT)$$

$$= [(1 + L_{CAM})/(K + L_{CAM} \cdot K')]/[(1 + L_{WT})/(K + L_{WT} \cdot K')]$$

If we assume that L for the WT receptor is negligibly small (e.g., $< 0.1\%$ of receptor is active in the absence of agonist) then this reduces to

$$\text{Affinity ratio} = (1 + L_{CAM})/(1 + L_{CAM} \cdot (K'/K)) \quad (2)$$

Also, if K'/K is fairly small as would be expected for an inverse agonist, the affinity ratio is nearly equal to $1 + L_{CAM}$. Thus an affinity ratio of 2 would mean that $L_{CAM} = 1$ and R and R^* are approximately equal so 50% of the receptor is in the R^* state without ligand present. A more complete examination of the behavior of this system without assumptions about the values of L or K'/K is shown (Fig. 8, bottom).

We also must examine the effect of an inverse agonist on receptor activity. We define the fraction of receptor that is active *in the absence of ligands* to be

$$f^* = R^*/(R + R^*)$$

$$= L/(1 + L)$$

To solve for L in terms of f^*, therefore

$$(1 + L) \cdot f^* = L$$

$$f^* + L \cdot f^* = L$$

$$f^* = L(1 - f^*)$$

$$L = f^*/(1 - f^*)$$

The fraction of receptor that is active also depends on ligand concentration and is

$$\frac{R^* + AR^*}{R + R^* + AR + AR^*} = \frac{L + L \cdot K' \cdot A}{1 + L + K \cdot A + L \cdot K' \cdot A}$$

Thus the amounts of receptor active at zero ligand and with saturating ligand are

Zero ligand	Saturating ligand
$L/(1 + L)$	$L \cdot K'/(K + L \cdot K')$

and the ratio of active receptor in the presence of ligand to active receptor in the absence of ligand is

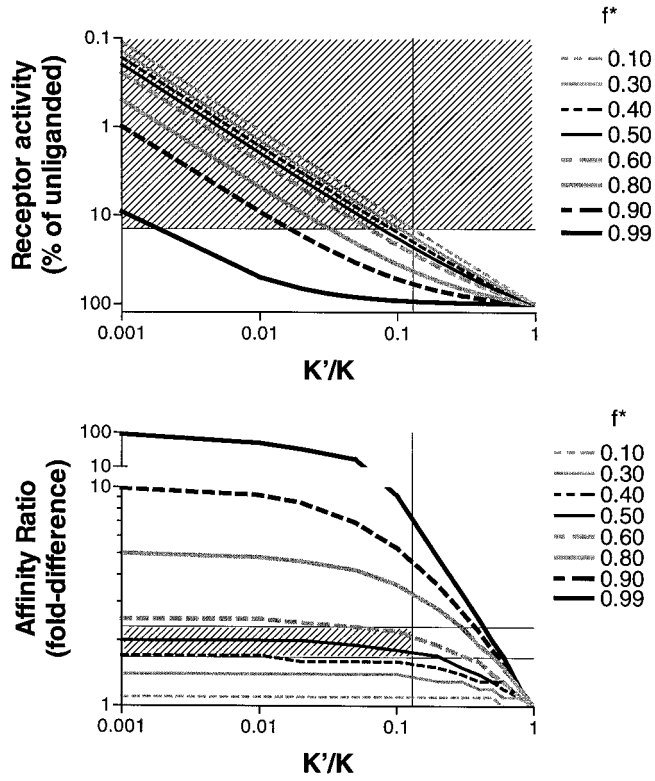

Fig. 8. Estimation of receptor activity and ratio of binding affinities for WT and CAM receptors. In the top panel, the amount of active receptor is decreased by an inverse agonist as a function of both the initial fraction of active receptor in the absence of ligand (f^*) and the affinity ratio for the active and inactive states of the receptor (K'/K). In the bottom panel, the apparent K_d increases for an inverse agonist with different values of K'/K as a function of f^*. The shaded areas indicate the ranges observed experimentally for rauwolscine and yohimbine. The top shaded area indicates values that represent a 7-fold decrease in receptor function (determined experimentally as a 7-fold increase in cAMP levels with rauwolscine or yohimbine). As can be seen by the theoretical lines, K'/K values must be <0.14 for the curves to reach this 7-fold decrease. The bottom shaded area includes this constraint ($K'/K < 0.14$, i.e., the area left of the vertical dashed line) plus the measured affinity ratio for rauwolscine and yohimbine (1.7–2.3). Theoretical curves that satisfy both of these constraints limit the value of f^* to 0.4 to 0.6.

[4] A less stringent assumption will also lead to the same final conclusions. If K is the same for WT and CAM receptors and L for the WT receptor is very low, then eq. 2 is still valid.

$$R^*_{A=\infty}/R^*_{A=0} = [L \cdot K'/(K + L \cdot K')]/[L/(1 + L)]$$
$$= [L \cdot (K'/K)/(1 + L \cdot (K'/K))]/[L/(1 + L)] \quad (3)$$

A coordinated examination of these relations is shown in Fig. 8. The top graph shows how the amount of active receptor would be decreased (eq. 3) by an inverse agonist as a function of both the initial fraction of active receptor (f^*) and the affinity ratio for the two states K'/K. A low K'/K means that the ligand binds preferentially to the inactive conformation. The bottom graph shows how the apparent K_d would increase for an inverse agonist with different values of K'/K as a function of f^*. A drug with no affinity difference between the R and R^* states (i.e., $K'/K = 1$) would show no change in apparent affinity and no reduction in receptor activity—as exemplified by idazoxan. As K'/K decreases, the K_d ratio increases with more active receptor (i.e., larger f^*) because the inverse agonist binds R^* less well. The shaded boxes indicate ranges observed experimentally for rauwolscine and yohimbine. Given the 7-fold increase in cAMP levels with rauwolscine, we would estimate that the amount of active receptor activity must be reduced by at least 7-fold. That number may be even larger if there is a threshold phenomenon (as noted under *Results*). Thus the values of f^* consistent with our experimental results range from approximately 0.4 to 0.6 meaning that approximately half the T373K CAM porcine α_{2A}-AR is in the active R^* state in the absence of ligand.

We can also put an upper limit on the fraction of WT receptor in the active state. We recently showed that the L1 cell line exhibits a receptor reserve of approximately 10^3 for UK-14,304 (Brink et al., 2000). Thus activation of ~0.1% of receptors can produce a 50% inhibition of cAMP accumulation. Based on the PTX and rauwolscine results, there appears to be little (conservatively <20%) inhibition of cAMP accumulation by the WT receptor in this line. This suggests that the amount of spontaneously active receptor may be as little as ~ 0.04% [i.e., <(20%/50%) × 0.1%] in the absence of agonist.

Acknowledgments

We thank Yang Hae Park for assistance with the UK-14,304 binding experiments.

References

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:**11687–11690.

Berg KA, Stout BD, Cropper JD, Maayani S and Clarke WP (1999) Novel actions of inverse agonists on 5-HT_{2C} receptor systems. *Mol Pharmacol* **55:**863–872.

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.

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.

Brink CB, Wade SM and Neubig RR (2000) Agonist-directed trafficking of porcine α_{2A}-adrenergic receptor signaling in CHO cells. *l*-Isoproterenol selectively activates G_s. *J Pharmacol Exp Ther* **294:**539–547.

Cayla C, Schaak S, Roquelaine C, Gales C, Quinchon F and Paris H (1999) Homologous regulation of the α2C-adrenoceptor subtype in human hepatocarcinoma, HepG2. *Br J Pharmacol* **126:**69–78.

Chen G, Way J, Armour S, Watson C, Queen K, Jayawickreme CK, Chen WJ and Kenakin T (2000) Use of constitutive G protein-coupled receptor activity for drug discovery. *Mol Pharmacol* **57:**125–134.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Chidiac P, Hebert TE, Valiquette M, Dennis M and Bouvier M (1994) Inverse agonist activity of β-adrenergic antagonists. *Mol Pharmacol* **45:**490–499.

Convents A, De Keyser J, De Backer J-P and Vauquelin G (1989) [^{3}H]Rauwolscine labels α_2-adrenoceptors and 5-HT_{1A} receptors in human cerebral cortex. *Eur J Pharmacol* **159:**307–310.

Costa T and Herz A (1989) Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci USA* **86:**7321–7325.

Costa T, Lang J, Gless C and Herz A (1990) Spontaneous association between opioid receptors and GTP-binding regulatory proteins in native membranes: Specific regulation by antagonists and sodium ions. *Mol Pharmacol* **37:**383–394.

Daunt DA, Hurt C, Hein L, Kallio J, Feng F and Kobilka BK (1997) Subtype-specific intracellular trafficking of α_2-adrenergic receptors. *Mol Pharmacol* **51:**711–720.

Eason MG, Kurose H, Holt BD, Raymond JR and Liggett SB (1992) Simultaneous coupling of α_2-adrenergic receptors to two G-proteins with opposing effects. Subtype-selective coupling of α_2 C10, α_2 C4, and α_2 C2 adrenergic receptors to G_i and G_s. *J Biol Chem* **267:**15795–15801.

Ernsberger P and Haxhiu MA (1997) The I_1-imidazoline-binding site is a functional receptor mediating vasodepression via the ventral medulla. *Am J Physiol* **273:**R1572–R1579.

Gerhardt MA (1992) *Coupling of the α_{2A}-Adrenergic Receptor to the G-Proteins Gi_2 and Gi_3*. Ph.D. Thesis, University of Michigan, Ann Arbor, MI.

Hein L, Limbird LE, Eglen RM and Kobilka BK (1999) Gene substitution/knockout to delineate the role of α_2-adrenoceptor subtypes in mediating central effects of catecholamines and imidazolines. *Ann NY Acad Sci* **881:**265–271.

Kenakin T (1995) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends Pharmacol Sc* **16:**232–238.

Kjelsberg MA, Cotecchia S, Ostrowski J, Caron MG and Lefkowitz RJ (1992) Constitutive activation of the a_{1B}-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation. *J Biol Chem* **267:**1430–1433.

Limbird LE, Speck JL and Smith SK (1982) Sodium ion modulates agonist and antagonist interactions with the human platelet α_2-adrenergic receptor in membrane and solubilized preparations. *Mol Pharmacol* **21:**609–617.

MacMillan LB, Hein L, Smith MS, Piascik MT and Limbird LE (1996) Central hypotensive effects of the α_{2a}-adrenergic receptor subtype. *Science (Wash DC)* **273:**801–805.

Meana JJ, Herrera-Marschitz M, Goiny M and Silveira R (1997) Modulation of catecholamine release by α_2-adrenoceptors and I_1-imidazoline receptors in rat brain. *Brain Res* **744:**216–226.

Nagaraja S, Iyer S, Liu X, Eichberg J and Bond RA (1999) Treatment with inverse agonists enhances baseline atrial contractility in transgenic mice with chronic β_2-adrenoceptor activation. *Br J Pharmacol* **127:**1099–1104.

Neubig RR, Gantzos RD and Brasier RS (1985) Agonist and antagonist binding to α_2-adrenergic receptors in purified membranes from human platelets: Implications of receptor-inhibitory nucleotide binding protein stoichiometry. *Mol Pharmacol* **28:**475–486.

Neubig RR, Gantzos RD and Thomsen WJ (1988) Mechanism of agonist and antagonist binding to α_2 adrenergic receptors: Evidence for a precoupled receptor-guanine nucleotide protein complex. *Biochemistry* **27:**2374–2384.

Neusy AJ and Lowenstein J (1989) Blood pressure and blood pressure variability following withdrawal of propranolol and clonidine. *J Clin Pharmacol* **29:**18–24.

Parma J, Duprez L, Van Sande J, Cochaux P, Gervy C, Mockel J, Dumont J and Vassart G (1993) Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature (Lond)* **365:**649–651.

Pauwels PJ and Wurch T (1998) Review: Amino acid domains involved in constitutive activation of G-protein-coupled receptors. *Mol Neurobiol* **17:**109–135.

Perez DM, Hwa J, Gaivin R, Mathur M, Brown F and Graham RM (1996) Constitutive activation of a single effector pathway: Evidence for multiple activation states of a G protein-coupled receptor. *Mol Pharmacol* **49:**112–122.

Rao VR, Cohen GB and Oprian DD (1994) Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature (Lond)* **367:**639–642.

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.

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. Extending the ternary complex model. *J Biol Chem* **268:**4625–4636.

Samama P, Pei G, Costa T, Cotecchia S and Lefkowitz RJ (1994) Negative antagonists promote an inactive conformation of the β_2-adrenergic receptor. *Mol Pharmacol* **45:**390–394.

Shenker A, Laue L, Kosugi S, Merendino, JJ Jr, Minegishi T and Cutler, GB Jr (1993) A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature (Lond)* **365:**652–654.

Tian WN, Duzic E, Lanier SM and Deth RC (1994) Determinants of α_2-adrenergic receptor activation of G proteins: Evidence for a precoupled receptor/G protein state. *Mol Pharmacol* **45:**524–531.

Wade SM, Lim WK, Lan KL, Chung D, Nanamori M and Neubig RR (1999) G_i activator region of α_{2A} adrenergic receptors: Distinct basic residues mediate G_i vs G_s activation. *Mol Pharmacol* **56:**1005–1013.

Weiss JM, Morgan PH, Lutz MW and Kenakin TP (1996) The cubic ternary complex receptor-occupancy model. III. Resurrecting efficacy. *J Theor Biol* **181:**381–397.

Wreggett KA and De Lean A (1984) The ternary complex model: Its properties and application to ligand interactions with the D_2-dopamine receptor of the anterior pituitary gland. *Mol Pharmacol* **26:**214–227.

Wurch T, Colpaert FC and Pauwels PJ (1999) G-protein activation by putative antagonists at mutant $Thr^{373}Lys$ α_{2A} adrenergic receptors. *Br J Pharmacol* **126:**939–948.

Send reprint requests to: Dr. Richard R. Neubig, Department of Pharmacology, 1301 MSRB III, 1150 W. Medical Center Dr., Ann Arbor, MI 48109-0632. E-mail: RNeubig@umich.edu