0026-895X/99/051005-09$3.00/0

MOLECULAR PHARMACOLOGY, **56:**1005–1013 (1999).

G_i Activator Region of α_{2A}-Adrenergic Receptors: Distinct Basic Residues Mediate G_i versus G_s Activation

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Received April 20, 1999; accepted July 30, 1999

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

ABSTRACT

The structural determinants of G protein coupling versus activation by G protein-coupled receptors are not well understood. We examine the role of two distinct basic regions in the carboxyl terminal portion of the third intracellular loop of the α_{2A}-adrenergic receptor to dissect these aspects of function. Changing three arginines to alanines by mutagenesis and stable expression in Chinese hamster ovary-K1 cells impaired the α_2-adrenergic receptor G_s-mediated stimulation of cyclic AMP (cAMP) accumulation, whereas G_i-mediated inhibition was normal. When two (B2) or three (B3) basic residues closer to transmembrane span 6 were mutated to alanine, normal ligand binding was observed, but G_i-mediated inhibition of cAMP accumulation showed 20-fold and 50-fold decreases in agonist potency for the B2 and B3 mutants, respectively. Surprisingly, a normal G_s response was seen for the B2 mutant, and the B3 mutant showed only a 6-fold decrease in agonist potency. Mutation of both the three alanines and B3 residues to alanines showed a 200-fold decrease in agonist potency for G_i-mediated inhibition of cAMP accumulation, whereas the G_s response was nearly completely eliminated. The three basic residues (which include the BB of the BBXXF motif) play a role as G_i activators rather than in receptor-G protein coupling, because high-affinity agonist binding is intact. Thus, we have identified three basic residues required for activation of G_i but not required for receptor-G protein coupling. Also, distinct basic residues are required for optimal G_i and G_s responses, defining a microspecificity determinant within the carboxyl terminal portion of the third intracellular loop of the α_{2a} adrenergic receptor.

G protein-coupled receptors (GPCRs) represent the most diverse superfamily of signal transduction molecules. More than 300 types and subtypes are known, not including the even more diverse olfactory receptor family. They activate heterotrimeric G proteins to mediate biological responses (Gudermann et al., 1997). GPCRs are involved in a broad range of signaling processes, including second messenger regulation, ion channel modulation, cell growth and differentiation, and cross-talk with tyrosine kinases and small-molecular-weight G proteins. The factors that determine the specificity of coupling of receptors to G proteins have not been fully worked out. The structure of the interface between receptor and G protein is one major factor that will be the focus of this study. Other factors, however, such as cellular localization (Neubig, 1998), other cellular proteins (Sato et al., 1995), and post-translational modifications of receptor (Daaka et al., 1997) or G protein, are also likely to contribute to this complex process.

In the 12 years since the cloning of the β adrenergic receptor (AR), much has been learned about the functional domains of GPCRs. Ligand binding sites for many GPCRs have been mapped, resulting in useful molecular models (Baldwin et al., 1997; Pogozheva et al., 1998). Little detailed structural information is available regarding receptor-G protein coupling, but the outlines of critical regions have been obtained by use of receptor chimeras, site-directed mutagenesis, and synthetic receptor peptides. In the studies employing chimeras and mutagenesis, both loss-of-function and gain-of-function alleles have been identified with respect to G protein coupling and activation (Strader et al., 1987; Kostenis et al., 1997). Much of this work, however, has not attempted to distinguish between the structural determinants of the *bind-*

This work was supported by National Institutes of Health Grant HL46417, the University of Michigan Multipurpose Arthritis Center (AR20557), and Natural Sciences and Engineering Research Council of Canada APP 207830-1998 (D.A.C.).

ABBREVIATIONS: GPCR, G protein-coupled receptor; AR, adrenergic receptor; i3n, amino-terminal end of the third intracellular loop; i3c, carboxyl-terminal end of the third intracellular loop; IBMX, isobutyl-1-methylxanthine; GppNHp, 5′-guanylyimidodiphosphate; R3, mutation of the RWRGR to AWAGA at residues 361 to 365; R3B3, clone in which receptors with both the R3 and B3 mutations were expressed; B2, mutation of 2 basic residues; B3, mutation of 3 basic residues; BBXXB, structural motif including basic (B) and non-basic (X) residues; CHO, Chinese hamster ovary; PIC, *p*-iodoclonidine; PTX, pertussis toxin; myr-α_{i1}, myristoylated recombinant α_{i1} subunit; WT, wild-type.

ing of G protein to the receptor and those responsible for the subsequent G protein *activation*.

Synthetic peptides from intracellular regions of GPCRs have also been used to identify possible G protein contact sites (König et al., 1989; Dalman and Neubig, 1991), and the results have been in reasonable agreement with mutagenesis studies [i.e., the second and third intracellular loops (i2 and i3, respectively) are most critical, but some contribution from i1 and the carboxyl tail may be present]. The rationale for this approach is that a peptide from a G protein-coupling domain of a receptor should itself bind to the G protein and either block or mimic receptor-mediated G protein activation. In contrast to mutagenesis studies, peptides may also provide information about coupling versus activation because some peptides block G protein activation by receptors, whereas others will activate the G protein by themselves. Peptides in the latter group represent candidate regions for the G protein activator portion of the receptor. Okamoto and Nishimoto first proposed that a BBXB or BBXXB motif was required for G_i activation (Ikezu et al., 1992; Okamoto and Nishimoto, 1992). Based on peptide structure-activity studies and existing literature, Wade et al. (1996) proposed a role for the i2 loop and the amino-terminal portion of the i3 loop (i3n) as coupling and specificity domains; the carboxy-terminal end of i3 (i3c) served as a G_i activator domain for the α_{2a}-AR. An arginine-rich region just amino-terminal to the BBXXB was identified as the likely G_i activator domain. The present work aimed to test this hypothesis in the context of the intact α_{2A}-AR.

Many GPCRs can activate G proteins from more than one family [see Gudermann et al. (1997) for review]. For example, angiotensin receptors activate G_i and G_q; thrombin receptors (proteinase activated receptor 1) activate G_i, G_q, and G_{12}; and thyrotropin-stimulating hormone receptors activate G proteins from all four families: G_i, G_s, G_q, and G_{12} (Laugwitz et al., 1996). In addition to activating G_i, the α_{2A}-AR has also been shown to stimulate adenylyl cyclase through the activation of G_s (Eason et al., 1992). Eason and Liggett examined the intracellular loop regions of the α_{2A}-AR by a chimeric approach and concluded that the i2, i3n, and i3c were all important for G_s coupling, whereas either i3n or i3c was sufficient for G_i coupling and activation (Eason and Liggett, 1995; Eason and Liggett, 1996).

In this report, we identify the basic residues in the i3c region of the α_{2A}-AR that are involved in G_i activation but not required for G protein-dependent, high-affinity agonist binding. In addition, we have defined a precise structural specificity in the coupling of the α_{2A}-AR to G_i and G_s within the small i3c region of the receptor. This type of microscopic specificity determinant may help explain recent observations of agonist trafficking or differential activation of distinct G proteins by two agonists acting at a single receptor (Kenakin, 1995; Berg et al., 1998) and will be important in evaluating the functional significance of structural changes that occur upon receptor activation.

Materials and Methods

Radiochemicals. [2-^{3}H]Adenine (21–25 Ci/mmol) was from Amersham Life Science (Arlington Heights, IL). *p*-[^{125}I]Iodoclonidine (2200 Ci/mmol), [^{3}H]yohimbine (74.5–78 Ci/mmol), and [^{35}S]guanosine 5′-3-*O*-(thio)triphosphate (1250 Ci/mmol) were from DuPont-New England Nuclear (Wilmington, DE).

Chemicals. Opti-MEM, Lipofectamine and geneticin (G-418) were from Gibco BRL (Gaithersburg, MD). Fluorescein-labeled antihemagglutinin epitope antibodies were from Boehringer Mannheim (Indianapolis, IN). Pertussis toxin was from List Biological Laboratories (Campbell, CA), forskolin from Calbiochem (LaJolla, CA), UK 14,304 was from Pfizer (Sandwich, England), clonidine was from Boehringer Ingelheim (Ingelheim, Germany), and oxymetazoline was from Schering Corporation (Bloomfield, NJ). Isobutyl-1-methylxanthine (IBMX), ATP, cAMP, 5′-guanylyimidodiphosphate (Gp-pNHp), and yohimbine were from Sigma (St. Louis, MO).

Construction of Mutant α_{2A}-Adrenergic Receptor Plasmids. The pCMV4-TAG α_2-AR construct was kindly provided by Dr. Lee Limbird (Vanderbilt University, Nashville, TN) (Keefer and Limbird, 1993). The single *Hin*dIII restriction site in the vector was destroyed by inserting a linker (AGCTAATT). Unique *Hin*dIII and *Nhe*I restriction sites were then introduced by overlap extension polymerase chain reaction, producing silent mutations of Ala359 and Lys376, respectively, yielding p$\alpha_2$tag H/N. The sequence of the polymerase chain reaction-generated fragment was verified by the University of Michigan DNA sequencing core facility using an Applied Biosystems DNA Sequencer. Mutagenic cassettes were used to introduce the subsequent mutations into the *Hin*dIII/*Nhe*I-digested p$\alpha_2$tag H/N vector by ligating complementary, annealed, 52-mer oligonucleotides containing the appropriate mutations, *Hin*dIII and *Nhe*I overhangs, plus a silent diagnostic *Nru*I restriction site when possible. When the *Nru*I site could not be included, the mutations were verified by sequencing.

R3 denotes the mutation of the RWRGR to AWAGA at residues 361 to 365 of the receptor (Fig. 1). Other basic residues in the membrane-proximal i3c region (residues 368–371) were also mutated to form B2 (BXAA) and B3 (AXAA) mutants. K370 and R371 represent the first two basic residues in the BBXXB motif (Okamoto and Nishimoto, 1992). R3B3 denotes the clone in which receptors with both the R3 and B3 mutations were expressed.

Cell Culture and Transfection. Chinese hamster ovary (CHO)-K1 cells were maintained in Ham's F-12 medium with 10% fetal bovine serum, 100 U/ml penicillin,and 100 μg/ml streptomycin at 37° in 5% CO_2. Selection for stable expression of mutants was maintained by the addition of 0.4 mg/ml G-418 (active).

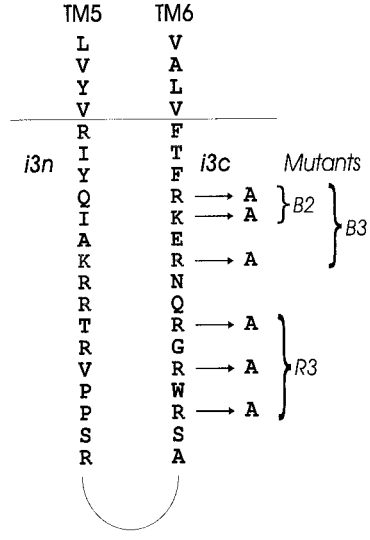

Fig. 1. Location of i3c mutations. Schematic diagram of the third intracellular loop of the porcine α_{2A}-AR receptor depicts the locations of the mutations of the positive charges in the carboxyl-terminal region of the loop. R3 denotes the mutation of the three membrane-distal arginines to alanines, and B2 and B3, respectively, denote mutation of the proximal two or three positive charges in the BXBB motif to alanines. TM5 and TM6 denote the fifth and sixth transmembrane regions of the receptor, respectively, and the horizontal line represents the inner surface of the plasma membrane.

CHO-K1 cells were cotransfected at a ratio of 5:1 with the α_{2A}-AR DNA (p$\alpha_2$tag H/N) and the pSV2neo plasmid (kindly provided by Dr. Jun Sadoshima, University of Michigan, Ann Arbor, MI). The DNA was added in Opti-MEM with 6 μl of Lipofectamine reagent per microgram of DNA for 24 h. Cells were returned to complete growth medium; 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 antihemagglutinin monoclonal antibody and single receptor-positive cells sorted into 96-well plates on a Coulter Elite ESP cell sorter. The individual cells were expanded and binding of [^{3}H]yohimbine determined as described below to evaluate receptor density.

CHO-K1 Membranes. Membranes were prepared as described previously (Wade et al., 1996), except that nuclei and undisrupted cells were first removed by pelleting for 10 min at 1000*g*. The final membrane pellets were resuspended in Tris/$MgCl_2$/EGTA buffer (50 mM Tris, 10 mM $MgCl_2$, 1 mM EGTA, pH 7.6), snap frozen and stored at −80°. Protein was determined by Bradford protein assay (Bradford, 1976).

Radioligand Binding Assays. Binding assays of the α_2-AR antagonist [^{3}H]yohimbine and the partial agonist *p*-[^{125}I]iodoclonidine (PIC) were performed on 2 to 5 μg of membrane protein in 96-well plates in a final volume of 100 μl as described previously (Neubig et al., 1985). For competition binding measurements, membranes were incubated with the indicated drugs in Tris/$MgCl_2$/EGTA buffer in the presence of 10 nM [^{3}H]yohimbine or 1 nM [^{125}I]PIC at room temperature for 30 to 60 min and filtered using a Brandel cell harvester. Nonspecific binding was defined by 10 μM the antagonist yohimbine or the partial agonist oxymetazoline, respectively.

Whole-Cell cAMP Accumulation. Whole-cell cAMP accumulation was determined in 24-well plates as described by Wong (1994). Briefly, cells were plated with 1 μCi/well [^{3}H]adenine and, where indicated, 100 ng/ml pertussis toxin (PTX) or 5 μg/ml cholera toxin, for 18 to 20 h before assay. Cells were washed once with Dulbecco's modified Eagle's medium. The assay was initiated by adding Dulbecco's modified Eagle's medium containing 1 mM IBMX, 30 μM forskolin, and the indicated drugs. Cells were incubated for 30 min at 37°C, and the reaction was terminated by aspirating the incubation medium and quenching with 5% trichloroacetic acid 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).

Expression and Purification of G Protein α_{i1} and $\beta\gamma$ Subunits. Myristoylated α_{i1} was expressed in *Escherichia coli* (BL21/DE3) and purified to homogeneity by column chromatography as described by Mumby and Linder (1994). Specific activity was 20 nmol/mg protein as determined by [^{35}S]guanosine 5′-3-*O*-(thio)triphosphate binding. To prepare $\beta\gamma$ subunits, bovine-brain G proteins were purified from cortex synaptosomal membranes (a gift from Dr. T. Ueda, University of Michigan, Ann Arbor, MI) by the method of Sternweis and Robishaw (1984) as modified by Kim and Neubig (1987). After activation for 30 min at 30°C with 20 μM $AlCl_3$, 10 mM $MgCl_2$, and 10 mM NaF, $\beta\gamma$ subunits were resolved from α as described by Katada et al. (1984) using a 100-ml phenyl-Sepharose column in place of the C7-Sepharose column. Purity was confirmed by SDS-polyacrylamide gel eletrophoresis. Activity of $\beta\gamma$ was determined by competition for fluorescein isothiocyanate-labeled α_{i1} binding to biotin-$\beta\gamma$ using fluorescence flow cytometry as described previously (Sarvazyan et al., 1998). Aliquots were snap frozen and stored at −80°.

Reconstitution of α_{2A}-Adrenergic Receptors with G protein. To deactivate endogenous G proteins, α_2-AR expressing CHO-K1 cells were incubated with 30 ng/ml PTX for 24 h before cell harvest and membrane preparation. To reconstitute high-affinity [^{125}I]PIC binding, membranes (3–4 nM α_2AR) were mixed with the indicated amounts of myristoylated recombinant α_{i1} subunit (myr-α_{i1})/$\beta\gamma$ in 50 mM Tris, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.1% cholate, pH 7.6. Samples were vortexed and kept on ice for 1 h before a 5-fold dilution into the radioligand binding assay buffer.

Data Analysis. Data were analyzed using the nonlinear least-squares methods in the computer program Prism (GraphPad Software, San Diego, CA). Statistical comparisons used unpaired *t* tests in InStat 3 (GraphPad Software, San Diego, CA). All IC_{50} values were converted to K_i values using the Cheng/Prusoff correction in Prism.

Results

In this study we examined the functional contribution of two distinct basic regions in the i3c region of the porcine α_{2A} receptor. Okamoto and Nishimoto (1992) proposed that a BBXXB motif is required for efficient G_i protein activation. In addition, we had identified a membrane-distal, arginine-rich sequence (RWRGR) corresponding to residues 361 to 365 that was required for stimulation of G_o GTPase by synthetic peptides (Wade et al., 1996). We therefore wanted to evaluate the role that these regions played in the context of the whole receptor.

Membrane-Distal Arginines Are Not Required for Activation of G_i but Are for G_s. Three mutant receptors were evaluated in which the membrane-distal (R3) or membrane-proximal (B2 and B3) positive charges were removed from the i3c loop by substitution with alanine (Fig. 1). The α_{2A}-AR mutants and a clone of the wild-type (WT) receptor were stably expressed in CHO-K1 cells at high levels of expression (10–36 pmol/mg protein; Table 1). Based on our earlier peptide data, which indicated a critical role for the arginines in G_o/G_i activation (Wade et al., 1996), we first evaluated the R3 mutant. [^{3}H]Yohimbine saturation binding to WT and R3 membranes showed K_d values that were not significantly different. B_{max} values were statistically significantly different but were within a factor of 2 of each other (Table 1). The R3 mutant receptor had a 3-fold higher affinity for the full agonist UK 14,304 than did the WT receptor in competition binding experiments ($p < .02$). Because of the high receptor expression level, which exceeds cellular G protein content, the higher affinity for UK 14,304 is an intrinsic property of the R3 mutant receptor and is not related to G protein coupling. Evidence for this conclusion includes a similar difference in affinities between R3 and WT in the pres-

TABLE 1

[^{3}H]Yohimbine binding to WT, R3, B2, B3, and R3B3 membranes

[^{3}H]Yohimbine binding to WT and mutant α_{2a}-AR in membranes was performed in 96-well plates for 30 min at room temperature. For saturation-binding experiments, 1 to 40 nM [^{3}H]yohimbine was used. Competition assays were done using 10 nM [^{3}H]yohimbine. Data represent the mean ± S.E. of three to five separate experiments performed in duplicate.

	[^{3}H]Yohimbine Binding		
	Saturation Binding		UK 14,304 Competition
	B_{max}	K_d	K_i
	pmol/mg protein	*nM*	*nM*
WT	19 ± 2	7.5 ± 2.2	83 ± 28
R3	10 ± 1*	4.9 ± 1.8	26 ± 4*
B2	36 ± 3*	8.3 ± 1.9	95 ± 18
B3	34 ± 4*	9.5 ± 3.2	78 ± 15
R3B3	34 ± 2*	6.3 ± 1.4	ND

*, results significantly different from WT, $p < .05$.
ND, not determined.

ence of GppNHp (28 versus 92 nM; $p < .001$, $n = 4$). Also, we did not detect separate high- and low-affinity binding components for any of the receptors in [^{3}H]yohimbine competition assays (data not shown). Studies of receptor-G_i protein coupling required the use of direct agonist binding, which selectively examines the high-affinity RG_i complex (see below).

The α_{2A}-AR has previously been shown to couple functionally to both G_i and G_s (Eason et al., 1992). In whole-cell measurements of cAMP accumulation in the presence of UK 14,304, we observed first a decrease in forskolin-stimulated cAMP accumulation at low agonist concentrations followed by an increase with higher concentrations of agonist for both the WT and R3 clones. However, the increase in cAMP with the R3 mutant was reduced compared with WT (Fig. 2, top). This reduction is not caused by decreased receptor expression because the inhibition of adenylyl cyclase is unaffected. As previously demonstrated by Eason et al. (1992), the inhibitory phase was blocked by PTX (Fig. 2, bottom), whereas the stimulatory phase was blocked by cholera toxin (data not shown). The increase in cAMP production by high concentrations of UK 14,304 is due to the α_{2A}-AR and not some other endogenous receptor, because it does not occur in stable cell lines transfected with the neomycin selection marker alone (Brink et al., 1999). To examine the stimulation alone, the inhibition was eliminated by pretreatment of cells with PTX (Fig. 2, bottom). The G_s-mediated increase in cAMP with the R3 mutant was shifted to the right 6-fold and the maximum response was reduced 40% compared with WT. In striking contrast, there was no difference in the EC_{50} values for G_i-mediated cyclase inhibition (0.2 nM). The 2-fold lower receptor density of the R3 mutant compared with WT receptors may cause the decreased maximum stimulation of cAMP levels. In the G_i response, the decreased receptor number may be offset by the intrinsic affinity of the agonist for the R3 receptor coupled with the known spare receptors for adenylyl cyclase inhibition in these CHO cells. These data are not consistent with our hypothesis that the three membrane-distal arginines represent the G_i activator, but they do indicate a role for these residues in G_s responses.

The BXBB Residues of the α_{2A}-AR Contribute Markedly to G_i Activation and Only Modestly to G_s Activation. We then asked if the BXBB sequence in the membrane-proximal region of i3c was involved in G_i activation. This

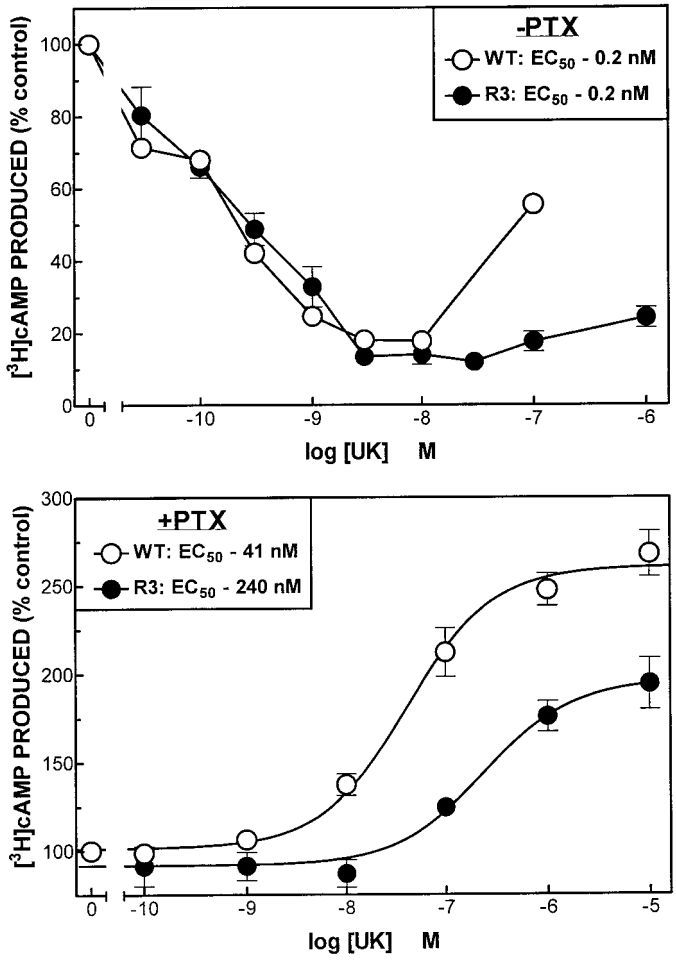

Fig. 2. α_2-AR Agonist-regulation of cAMP in WT and R3 cells. Whole-cell [^{3}H]cAMP accumulation measurements were performed on WT (○) or R3 (●) cells that had been pretreated with (bottom) or without (top) PTX. Cells were incubated with increasing concentrations of UK 14,304 for 30 min at 37°C in the presence of 1 mM IBMX and 30 μM forskolin. Data are the mean ± S.E. of three or four separate experiments performed in duplicate.

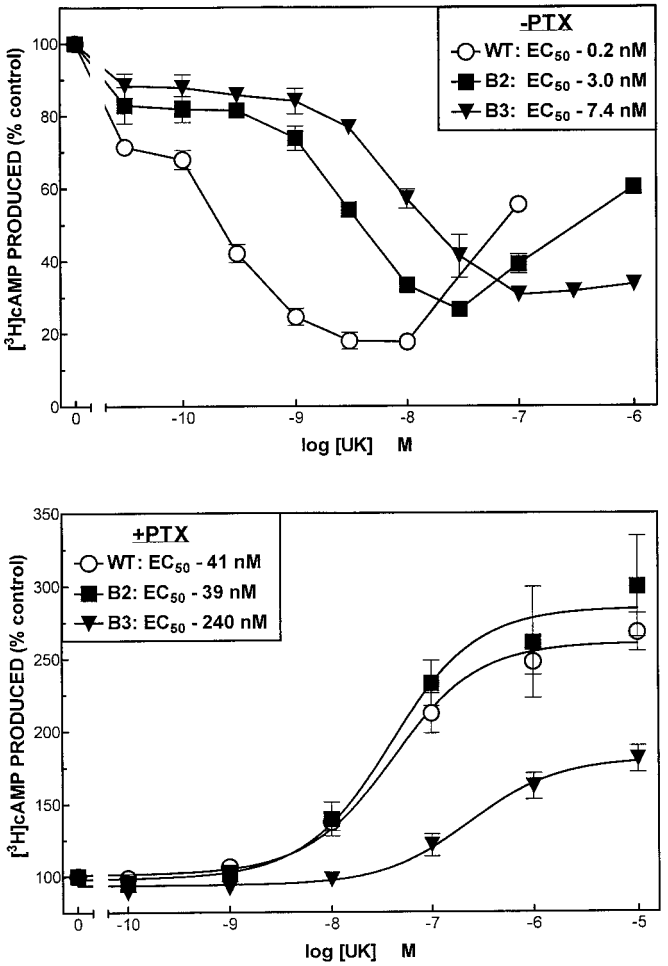

Fig. 3. Agonist-mediated cAMP regulation in WT, B2, and B3 mutants. Whole-cell [^{3}H]cAMP accumulation assays on WT (○), B2 (■), or B3 (▼) cells were carried out for 30 min at 37°C in the presence of 1 mM IBMX, 30 μM forskolin, and increasing concentrations of UK 14,304 with (bottom) or without (top) PTX pretreatment. Data are the mean ± S.E. of three or four separate experiments performed in duplicate.

sequence overlaps with the BBXXB proposed by Okamoto and Nishimoto (1992) to be a G_i activator. The BBXXB hypothesis has never been tested directly in the context of an intact G_i-coupled receptor. In [^{3}H]yohimbine saturation-binding experiments, the WT, B2, and B3 clones had similar K_d values with B_{max} values of 19, 36 and 34 pmol/mg protein, respectively (Table 1). All three receptors also displayed similar affinities for the agonist UK 14,304 in competition binding experiments (Table 1).

In whole-cell cAMP accumulation assays, we again observed an inhibition of forskolin-stimulated adenylyl cyclase activity at low concentrations of agonist, followed by an increase at higher UK 14,304 concentrations. In striking contrast to the R3 mutation, UK 14,304 dose-response curves for G_i-mediated inhibition were shifted to the right by 20-fold and 50-fold for the B2 and B3 mutants, respectively (Fig. 3, top). Similar results were seen in preliminary studies with three additional WT cell lines and five additional B2 and B3 cell lines. Thus, removal of the three membrane-proximal basic residues (BXBB) dramatically reduced the ability of the α_{2A}-AR to activate G_i. This was not attributable to a change in K_d for UK 14,304 because there was no decrease in agonist affinity in competition binding studies (Table 1). The fact that the decreased G_i activation is manifested as an increase in IC_{50} value rather than a decrease in the percentage of inhibition is because of the substantial receptor reserve for α_2-AR-mediated inhibition of cAMP accumulation in these high expressing cells (Brink et al., 1999). Thus these three basic residues play a major role in the G_i activation.

Thinking that the three arginines might contribute to the residual inhibition of cAMP accumulation in the B3 mutant, we prepared the combination R3 and B3 mutant (R3B3). Three different cloned cell lines tested still showed inhibition of forskolin-stimulated cAMP accumulation, however the UK 14,304 dose response curves were shifted further to the right (Table 2).

Interestingly, G_s activation by the mutant α_{2A}-ARs showed a different pattern of effects from that of G_i. Pertussis toxin pretreatment of cells revealed a pure stimulation of cAMP accumulation in all three clones (Fig. 3, bottom). Although both the WT and B2 mutants displayed identical G_s-mediated cAMP increases, stimulation of cAMP accumulation by the B3 mutant was shifted to the right approximately 6-fold and the maximal response was reduced by 50%. G_s-mediated cAMP increases were reduced nearly 90% by the R3B3 mutation (Table 2). Thus the B2 mutation results in a pure disruption of G_i responses and the R3 mutation a pure effect on G_s responses, whereas the B3 mutation reduces both G_i and G_s signaling.

TABLE 2

Functional Parameters of WT, R3, B2, B3, and R3B3 coupling to G_i and G_s

[^{3}H]cAMP accumulation assays were performed on whole cells that had been pretreated with (G_S coupling) or without (G_i coupling) PTX. Cells were incubated with increasing concentrations of UK 14,304 for 30 minutes at 37° in the presence of 1 mM IBMX and 30 μM forskolin. Maximum responses and EC_{50} values were determined from dose response curves. Data are from three or four separate experiments performed in duplicate.

	G_i Coupling		G_S Coupling	
	Max Inhibition	EC_{50}	Max Stimulation	EC_{50}
	%	*nM*	*% over control*	*nM*
WT	85	0.15	160	41
R3	87	0.2	100	240
B2	82	3.0	180	39
B3	71	7.4	80	240
R3B3	64	28	20	190

The BXBB Region Is Required for G_i Activation but Not for G_i Coupling by the α_{2A}-AR. There are two possible mechanisms whereby the B2 and B3 mutations could disrupt α_{2A} receptor-mediated activation of G_i. The mutations could either disrupt the physical RG interaction or they could prevent G protein activation, which occurs subsequent to the initial RG coupling. Because agonist competition curves did not reveal RG coupling, we directly measured the high-affinity agonist binding. This probes only the coupled form of the receptor and has been used extensively as a measure of α_2-AR–G_i interactions (Neubig et al., 1985; Kim and Neubig, 1987; Neubig et al., 1988). In saturation binding assays with the partial agonist [^{125}I]PIC, WT, B2, and B3 membranes all exhibited high-affinity binding, which was decreased to similar levels by 10 μM GppNHp (Table 3 and Fig. 4). The K_d value for [^{125}I]PIC at the B3 mutant receptor was slightly higher than its K_d value for WT (1.4 nM us 0.8 nM, Table 3). However, for all three receptors, the K_d values for [^{125}I]PIC, in the absence or presence of 10 μM GppNHp, were within a factor of 2 of each other, indicating that RG coupling was preserved. High-affinity α_2-AR agonist binding with either the partial agonist [^{125}I]PIC or the full agonist [^{3}H]UK 14,304 is completely eliminated by PTX pretreatment of the cells (data not shown). This indicates that the high-affinity binding only probes receptor coupling to endogenous G_i family proteins and not coupling to G_s.[1] Although WT membranes expressed only about half as much receptor as B2 and B3 membranes, as assessed by [^{3}H]yohimbine saturation binding (19 pmol/mg protein versus 36 and 34 pmol/mg protein, respectively), high-affinity agonist binding in WT membranes was 3.2 pmol/mg protein compared with 1.9 and 1.7 pmol/mg protein in B2 and B3 membranes, respectively. The significance of these B_{max} differences is not clear, although a limited amount of G_i may contribute to the small fraction of receptor that is able to bind agonist with high affinity (see also reconstitution data below).

As a second approach to characterize the nature of the interaction between the mutant receptors and G protein, we looked at the concentration dependence of GppNHp-induced inhibition of high-affinity agonist binding (Fig. 5). The IC_{50} values for GppNHp to reduce [^{125}I]PIC binding were 200, 15 and 2.1 nM, respectively, for WT, B2, and B3 membranes. If the ability of GppNHp to reduce agonist binding is inversely related to the affinity of the RG complex, then these results suggest that the B3 does not couple well to the G protein. Because nucleotide triphosphate binding to G protein is stimulated by agonist activation, however, this may actually represent an alternative measure of G protein activation rather than a measure of the initial RG affinity.

Finally, as a third measure to determine whether the mutants were impaired in their ability to couple to G protein, PTX-treated membranes expressing the mutant receptors were reconstituted with purified G_i protein and assayed for high-affinity agonist binding. The rationale for this experi-

[1] The endogenous G_i-family proteins present in CHO cells are G_{i2} and G_{i3} (Gerhardt and Neubig, 1991). Both contribute to high affinity binding of and adenylyl cyclase inhibition by PIC and UK 14,304, although G_{i2} appears to play the larger role (Gerhardt and Neubig, 1991).

ment is that the similar PIC binding affinities for the WT, B2, and B3 receptors could be related to limited G protein rather than similar coupling affinities. To test this, we wanted to see how the different receptors coupled in the face of a range of G protein concentrations. Both the B2 and B3 mutants were able to couple effectively to purified $\alpha_{i1}\beta\gamma$ subunits (Fig. 6). With the addition of extra G protein, the binding of the agonist [^{125}I]PIC (7–12 pmol/mg protein) to PTX-treated membranes is increased significantly above that in control membranes before PTX treatment (2–3 pmol/mg protein; see Table 3). [^{125}I]PIC binding for the two mutants was similar to that of WT at receptor/G protein molar ratios of 1:10 and 1:50; however, binding to the B3 mutant receptor was slightly less than for WT and B2 at a molar ratio of 1:200. Thus, the reconstitution results also show nearly normal RG_i coupling.[2]

Discussion

In this report, we define the G_i activator role of positively charged amino acids in the i3c region of the α_{2A}-AR and have also elucidated regions that contribute differentially to G_i and G_s responses activated by that receptor. Many previous mutagenesis studies have utilized chimeras that predominantly test sites of *specificity* between the two proteins. This approach may miss sites that have a particular function if that function is conserved between the two systems (as an activator motif might be). With a series of alanine substitutions and combined functional, binding, and reconstitution methods, we have identified a G_i activator region of the α_{2A}-AR.

G_i Activator Region. At least two sequences within the α_{2A}-AR have been proposed as potential G_i activators on the basis of studies with synthetic peptides. Okamoto and Nishimoto (1992) identified a BBXB or BBXXB motif. In the case of the α_{2A}-AR i3c loop, Ikezu et al. (1992) suggested that it was present in a modified form as BBXXF. We proposed that three arginines farther in the amino-terminal direction in the i3c (RWRGR, residues 361–365) were mainly responsible for G_o and G_i activation based on peptide structure-activity relations (Wade et al., 1996). The results of our present study clearly demonstrate that the RWRGR sequence is not involved in G_i activation in the context of the intact receptor; however, it does play a role in G_s activation (see below). The BB pair of residues in the BBXXF sequence is very important in G_i activation, although an additional basic residue two amino acids upstream also seems to contribute significantly to G_i activation. The evidence that this BXBB sequence is a G protein activator rather than just being involved in the G protein binding derives from a comparison of the *G protein activation* determined from adenylyl cyclase assays with measures of *G protein coupling* determined by the guanine nucleotide-regulated binding of the α_{2A} agonist [^{125}I]PIC. Because high-affinity agonist binding is dependent on both the affinity of receptor for G protein and the amount of G protein present, we also used reconstitution methods to compare PIC binding of the WT and mutant α_{2A}-ARs in the presence of varying amounts of added G_i. For the B2 mutant, there was no significant change in agonist binding affinity. For the B3 mutant, there was a slight decrease in the binding of PIC (less than 2-fold), whereas the efficiency of G_i activation was reduced 50-fold, clearly showing a selective effect on G protein activation.

One possible explanation of the disruption of responses with retained high-affinity binding could be that different G

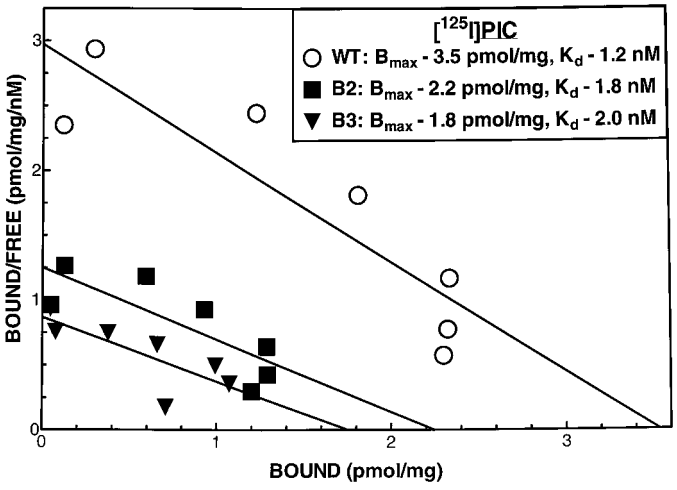

Fig. 4. Scatchard analysis of [^{125}I]PIC binding to WT, B2, and B3 membranes. Saturation binding to WT (○), B2 (■), or B3 (▼) membranes was performed using 0.1 to 4 nM [^{125}I]PIC for 30 to 45 min at room temperature. Nonspecific binding, defined in the presence of 10 μM oxymetazoline, represented less than 10% of the total binding and was subtracted. Values are the mean of four experiments, each performed in duplicate. A summary of binding parameters is presented in Table 3.

[2] Attempts to reconstitute high affinity binding with bacterially expressed α_s (gift of Dr. Ron Taussig) plus brain $\beta\gamma$ were unsuccessful. Negative data are difficult to interpret but this may be due to the relatively inefficient coupling of the α_2-AR with G_s versus G_i. Evaluation of receptor reserve for G_i versus G_s indicates that G_i couples to receptor 100 times better than G_s (Brink et al., 1999). Thus our conclusions about physical RG coupling apply to R-G_i coupling and we can not make any conclusions about physical R-G_s coupling, only functional coupling.

TABLE 3

Binding parameters of WT, B2, and B3 stable lines

Saturation binding to membranes from CHO-K1 cells expressing WT and mutant α_{2a}-AR was performed in 96-well plates. Membranes were incubated with 1 to 40 nM [^{3}H]yohimbine or 0.1 to 4 nM [^{125}I]PIC for 30 to 45 min at room temperature. [^{125}I]PIC binding was done in the presence or absence of 10 μM GppNHp. Data represent the mean ± S.E. of four or five separate experiments performed in duplicate.

	Saturation Binding				
	[^{3}H]Yohimbine	[^{125}I]PIC			
	B_{max}	B_{max}		K_d	
		−GppNHp	+GppNHp	−GppNHp	+GppNHp
	pmol/mg protein			*nM*	
WT	19 ± 2	3.2 ± 0.7	1.5 ± 0.6	0.8 ± 0.1	1.4 ± 0.3
B2	36 ± 3	1.9 ± 0.5	1.2 ± 0.5	1.2 ± 0.3	2.2 ± 0.7
B3	34 ± 4	1.7 ± 0.7	0.8 ± 0.3	1.4 ± 0.3	1.9 ± 0.3

proteins are involved in the two processes. We don't think that different G proteins could explain our findings. We previously showed, using subtype-specific antibody inhibition, that G_{i2} and G_{i3} are the primary G proteins involved in both the high-affinity binding and adenylyl cyclase inhibition by UK 14, 304 and PIC (Gerhardt and Neubig, 1991). Thus, the full and partial agonists seem to use the same G_i family members.

Another piece of evidence that UK 14,304 and PIC don't result in selective coupling to different G proteins similarly derives from their functional activity for G_i and G_s. Although PIC is a partial agonist and does not activate the α_{2A}-AR well, it results in similar relative stimulation of G_i and G_s. The relative intrinsic activities of PIC compared with UK 14,304 are 0.28 for G_i and 0.30 for G_s in regulation of cAMP accumulation (C. B. Brink, R. R. Neubig, in preparation).

For many other GPCRs, in which mutagenesis of the intracellular loop regions has been done, parallel losses of G protein activation and high-affinity GTP-sensitive agonist binding have been seen, indicating that these mutations are important for coupling to G protein as well as for activation. There have, however, been several mutagenesis studies in which there is dissociation between the changes in binding and response. Most are with receptors coupled to G proteins other than G_i. One of the first reports was of β_2-AR mutants which showed high-affinity agonist binding, typically associated with G protein coupling, but no GTP shift in agonist binding or G_s activation (Strader et al., 1987). This phenotype was seen with small deletions in both the i3n and i3c region of the β_2-AR. For i3c, the deletion (Δ258–270) encompassed both a BXBB sequence and positive charge in the amino-terminal direction to the BXBB. Because the GTP shift was abolished, this may represent a disruption in G protein coupling because of structural changes from the deletion. Lee et al. (1996) found a role for positive charges in the i3c region in G_q coupling of the muscarinic m1 receptor. Alanine substitution of positive charges in the i3n region did not disrupt either G_q activation or high-affinity agonist binding, similar to the results of Cheung et al. for the β_2-AR and G_s (Cheung et al., 1992). In contrast, mutation of the positive charges in the i3c region of the m1 receptor significantly disrupted PLC activation. Alanine substitution for either of the first two basic residues in the BBXXB region led to decreases in response with some retention of high-affinity ligand binding and the GTP shift. Removing both basic residues, which is equivalent to our B2 mutant, disrupted both G_q activation and agonist binding, in contrast to our lack of effect on agonist binding. In the case of rhodopsin, Ernst et al. (1995) found two mutants, CD r140–152 and EF 237–249, which bound transducin but failed to induce release of GDP. The latter mutant includes lysine 248 (which aligns with the second B in the BXBB region), which we have found to be important in the α_{2A}-AR, and also deletes lysine 245 (which would be one residue in the amino-terminal direction to our first B). Thus, the functional behavior of this rhodopsin mutant is very similar to that of our B3 mutant. These data for G_q and transducin as well as our current data support the importance of positive charges in i3c in receptor-mediated activation of G proteins.

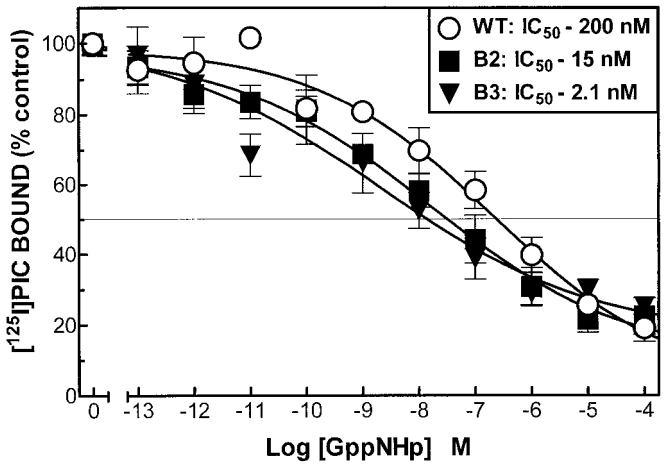

Fig. 5. [^{125}I]PIC binding to WT, B2, and B3 membranes in the presence of GppNHp. WT (○), B2 (■), or B3 (▼) membranes were incubated with 1 nM [^{125}I]PIC in the presence of increasing concentrations of GppNHp for 45 min at room temperature, then filtered as described. Data were fit to a sigmoidal dose-response curve with variable Hill slope and are the mean ± S.E. of four separate experiments performed in duplicate.

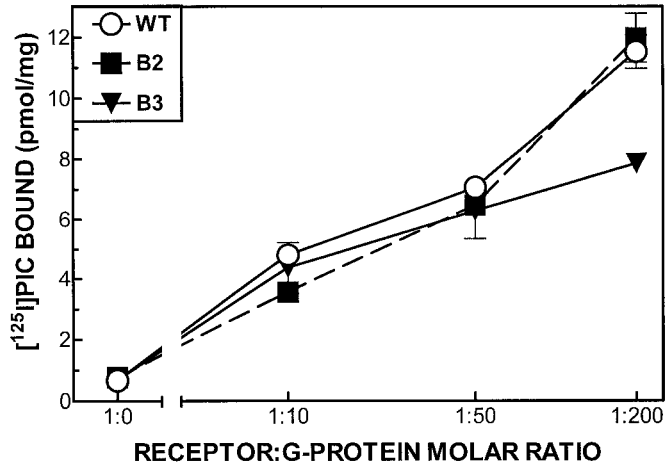

Fig. 6. Reconstitution of WT, B2, and B3 membranes with G protein. Membranes expressing WT (○), B2 (■), or B3 (▼) α_2-AR were reconstituted with the indicated amount of myr-αi1/$\beta\gamma$ and assayed for [^{125}I]PIC binding (1 nM). Specific binding is expressed as picomoles per milligram of protein and the receptor/G protein ratio is plotted on a log scale. Data represent the mean ± S.E. of five experiments, each performed in triplicate.

Some other data do not fit with the BXBB being the sole G_i activator. This includes a deletion mutant in the middle of the m4 muscarinic receptor i3 loop, which caused loss of G_i activation but retention of high-affinity agonist binding (Van Koppen et al., 1994). Interestingly, this deletion includes the first B of the BXBB region, which we found to be important in α_{2A}-AR-mediated G_i activation. However, the residue at the other end of the deletion is also an arginine, which would reconstitute the BXBB sequence. Thus, the BXBB is not sufficient for G_i activation. Perhaps in this case, the cluster of four basic residues just amino-terminal of the BXBB contributes to $G_{i/o}$ activation or the junction formed by removal of the deleted segment may cause a conformational change, which prevents the BXBB from appropriately contacting G_i to produce activation. Liu et al. (1995) found four residues, VTxxIL, in the i3c region of the m2 muscarinic receptor, which would permit G_i responses by the G_q-coupled m3 muscarinic receptor. The reciprocal change to AAxxLS conferred G_q activation on the m2 receptor and destroyed the G_i response. These mutations, however, weren't examined to distinguish G protein coupling from activation so these structural fea-

tures should be considered as specificity determinants rather than established activator regions.

G_i/G_s Coupling Specificity. The ability of α_2-AR to stimulate adenylyl cyclase as well as to inhibit it has been demonstrated by several groups (Eason et al., 1992; Pepperl and Regan, 1993; Nasman et al., 1997). Direct activation of G_s is the mechanism because the stimulation: 1) occurs in plasma membranes, 2) is insensitive to PTX, 3) is sensitive to cholera toxin, and 4) is reduced by anti-G_s antisera (Eason et al., 1992; Nasman et al., 1997). Although there are some discrepancies regarding which subtype of the α_2-AR is most effective in coupling to G_s (Eason et al., 1992; Pepperl and Regan, 1993; Nasman et al., 1997), our data support the conclusion that in mammalian cells the α_{2a}-AR produces significant G_s activation when the receptor is expressed at high levels (i.e, >1 pmol/mg protein).

The structural basis for the dual coupling to G_i and G_s has been examined by two groups. Eason and Liggett (1996) found that substitution of 5-HT1a sequence in the i2, i3n, or i3c region of the α_{2a}-AR resulted in loss of G_s stimulation, whereas G_i activation was essentially unchanged. Interestingly, they concluded that the i3c was not necessary for G_i activation, because substitution of β_2-AR sequence in that region alone did not disrupt G_i responses. Based on our alanine substitution data, it seems likely that the β_2 sequence KEHK is able to substitute for the REKR in the α_{2a}-AR, a possibility noted by Eason and Liggett (1996). G_s coupling by the α_{2a}-AR was further probed with deletion and more localized substitution of the i3n region with 5HT1a sequence (amino acids 218–228), which also disrupted G_s responses (Eason and Liggett, 1995). They identified a relatively small part of i3n as critical in G_s coupling. Nasman et al. (1997) found that cAMP accumulation in intact Sf9 cells was more pronounced for α_{2b}- than α_{2a}-ARs and that the i2 loop provided this specificity with a S134A/L143S mutation contributing in part. In their study, however, it was largely specificity that was examined, because agonist-binding studies were not undertaken. Thus, our results provide new information about the microspecificity within a single receptor domain (i3c) in which K370/R371 are critical for G_i activation, the arginine residues at 361, 363, or 365 are involved in G_s activation, and R368 contributes to both G_i and G_s responses.

As in the tyrosine kinase system, where specific phosphorylated tyrosines contribute to differential effector coupling (Malarkey et al., 1995), this type of microscopic specificity determinant may help explain recent observations of agonist trafficking or differential activation of distinct G proteins by two agonists acting at a single receptor (Kenakin, 1995; Berg et al., 1998). A full understanding of the structural basis of receptor specificity for G proteins will depend on the identification, at the single amino acid level, of the determinants for activation of different G protein types. Indeed, such a molecular level of specificity may be important in regulation of G protein specificity at a post-translational level also (Daaka et al., 1997). Further definition of the molecular structures that determine receptor-G protein activation and specificity will be essential to interpret structural information from these proteins as it becomes available.

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