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The Third Extracellular Loop of the β_2-Adrenergic Receptor Can Modulate Receptor/G Protein Affinity

MING-MING ZHAO,[1] ROBERT J. GAIVIN, and DIANNE M. PEREZ

Department of Molecular Cardiology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, Ohio 44195

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ABSTRACT

Chimeric receptors of the β_2-adrenergic receptor in which the extracellular loops were replaced with the corresponding amino acids of the α_{1a}-adrenergic receptor were generated to measure changes in α_1-antagonist affinity. Although no changes in α_1-antagonist affinity were measured in the β_2/α_{1a} chimeras, a decreased IC_{50} (10-fold) for agonists as compared with wild type β_2 control was found because of the replacement of the third extracellular loop (EX3). These agonist high affinity changes were because of a greater proportion of high affinity sites (2-fold) that were convertible to low affinity sites with guanosine 5′-3-*O*-(thio)triphosphate. Adenylate cyclase activity evoked by the EX3 chimera showed commensurate increases in the basal signal transduction as well as the isoproterenol-stimulated potency, suggesting constitutive activity. However, unlike other constitutively active adrenergic receptor mutants in which the mutation causes G protein-independent changes, the mechanism of the EX3 chimera seems to be attributable to a greater ease with which the active ternary complex is formed because of a higher affinity/coupling of the G protein. Although the changes because of EX3 are indirect and most likely affect helical packing, they support an emerging hypothesis that G protein-coupled receptors have evolved their structure-function relationships to constrain the receptor in an inactive state.

Many hormone receptors mediate their intracellular responses by interacting with one or more of a family of G proteins. The process of receptor activation and G protein coupling is described by the widely accepted revised TCM (Samma *et al.*, 1993). In this allosteric model, the active conformation of the native receptor, which leads to signaling, is the cornerstone of the ternary agonist-receptor-G protein complex. Without agonist present, the model predicts spontaneous receptor isomerization between inactive (R) and the active conformation (R*), with equilibrium under native conditions shifted toward R. At any given time, even though most receptors reside in R, a small population will reside in R*, permitting formation of the HR*G protein complex that causes effector activation. Receptor mutations that induce an agonist-independent shift in isomerization toward the R* conformation are termed CAMs and couple to and evoke second messenger responses greater than the WT receptor in the absence of agonist.

Receptor-mediated activation of G proteins involves the exchange of complexed GDP for GTP and the GTP-bound form of the G protein interacts with the specific effector system. The receptor-G protein complex has a higher affinity for agonists than does the free receptor, resulting in the appearance in membrane preparations of a high affinity agonist site in addition to low affinity sites characteristic of the free or uncoupled receptor. Addition of GTP or GTP analogs, which uncouple the receptor-G protein complex, can convert the high affinity site to the low affinity site. Although receptor activation models and GTP-evoked shifts in affinity (which are dependent on receptor/G protein ratios) have not been correlated (i.e., 30% high affinity sites does not mean a 30% "active" or R* population), the revised TCM model assumes that both hormone and G protein bind with greater affinity to the R* form. Given this condition, there is cooperativity in binding of the G protein and hormone to the receptor which explains the GTP shift and, therefore, can be used as a relative but not absolute measure of the active ternary complex, HR*G.

Of the G protein-coupled receptors, the β_2-AR is perhaps the best characterized and most widely used to formulate activation theories. They display the characteristic G protein-coupled receptor topography of seven membrane-spanning domains. They form a family of related receptors (α_1, α_2, β_1, β_2, and β_3 subtypes) that mediate the sympathetic actions

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[1] Current affiliation: Division of Hematology, Vanderbilt University, Nashville, TN 37232.

ABBREVIATIONS: AR, adrenergic receptor; CAM, constitutively active mutation; EX, extracellular loop; [^{125}I]CYP, iodocyanopindolol; TCM, ternary complex model; TM, transmembrane; WT, wild-type; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; ANOVA, analysis of variance; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

of epinephrine and norepinephrine. Whereas the agonist-binding pocket for these receptors is formed within a hydrophilic pocket formed by the circular array of the TM domains, we have obtained evidence that members of the α_1-adrenergic family may bind antagonists partly on the extracellular surface of the receptor (Zhao *et al.*, 1996). To explore this hypothesis further, we made a series of chimeras in which we replaced the extracellular loops of the β_2-AR with the corresponding loops of the α_{1a} subtype, thinking that we could impart to the β_2-AR a higher affinity for α_1-antagonists. Although we did not observe this phenotype, we serendipitously found that replacement of the third extracellular loop imparted to the β_2-AR a higher binding affinity for agonists, an increased potency, and an agonist-independent higher basal activity of adenylate cyclase. Unlike other previously characterized CAMs, where the high affinity binding of agonists was intrinsic to the receptor and independent of G protein modulation, the β_2/α_{1a} chimeric phenotype of constitutive activity seems to be attributable to a higher affinity/coupling of the G protein for the receptor promoting the formation of the active ternary complex. This suggests a possible role of the third extracellular loop of the β_2-AR in controlling receptor/G protein affinity; however, this is most likely caused indirectly by influencing the arrangement of TM 6 and 7 in the membrane bilayer.

Experimental Procedures

Construction of β_2/α_{1a} chimeric receptors. The constructs used were the synthetic gene of the hamster lung β_2-AR (Noda *et al.*, 1994) or the rat α_{1a}-AR cDNA (Perez *et al.*, 1994). The synthetic β_2-AR has unique restriction sites spaced approximately 50 base pairs apart to facilitate mutagenesis via oligonucleotide-cassette replacement. The synthetic gene displays WT pharmacology and signal transduction properties of the β_2-AR cDNA (Noda *et al.*, 1994). Generation of the β_2/α_{1a} chimeric receptors was accomplished via double restriction digestion of the synthetic WT β_2-AR, removal of the cassette by agarose gel electrophoresis, ligation of the annealed oligonucleotide replacement cassette containing the appropriate sticky ends, and encoding of the rat α_{1a} sequence in the corresponding region to the β_2-AR as described previously (Noda *et al.*, 1994). cDNAs were sequenced by the dideoxy method (Sequenase; Amersham, Arlington Heights, IL) to confirm the chimeras. Fig. 1 shows the amino acids that were replaced in the β_2-AR with the rat α_{1a} sequence (in *black*). Location of the start and stop points was determined by the location of the restriction sites in the synthetic gene. The designations, EX1, EX2, and EX3 (see Table 1) correspond to β_2/α_{1a} chimeric receptors in which the EX1, EX2, or EX3 regions were replaced individually with α_{1a}-residues. The EX1+2+3 designation refers to the β_2-AR with replacement of all three extracellular loops with α_{1a}-residues.

Cell culture and transfection. COS-1 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. cDNAs encoding the WT β_2-AR and various chimeras were subcloned into the mammalian expression vector pMT2′, as previously described (Perez *et al.*, 1991). Plasmid DNA, purified by Wizardprep (Fisher Scientific, Pittsburgh, PA) and Bio-Gel A-50m column chromatography (Bio-Rad, Hercules, CA), was used to transfect cells. Transient expression in COS-1 cells was accomplished by the DEAE-dextran method (Sambrook *et al.*, 1989) using the same mastermix of reagents on the same day with all pertinent constructs on equalized cell numbers. Efficiency of transfection is 20% as determined by β-galactosidase staining. Cells were harvested 60 hr post-transfection.

Membrane preparation and radioligand binding. COS-1 membranes were prepared as previously described (Perez *et al.*, 1991). The protein concentration was measured using the method of Bradford (1976). The ligand-binding characteristics of the expressed receptors were determined in a series of radioligand binding studies using the β_2-antagonist radioligand [^{125}I]CYP as previously described at 37° (Noda *et al.*, 1994). Competition reactions (total volume, 0.25 ml) contained 20 mM HEPES, pH 7.5, 1.4 mM EGTA, 12.5 mM $MgCl_2$, 150 pM [^{125}I]CYP, COS-1 membranes, and increasing amounts of unlabeled ligands known to interact with β_2-ARs. Nonspecific binding was determined in the presence of 10^{-5} M propranolol. Reactions were stopped by the addition of cold HEPES buffer and were filtered onto Whatman GF/C glass fiber filters with a Brandel cell harvester (Brandel, Gaithersburg, MD). Filters were washed five times with HEPES buffer, and bound radioactivity was determined using a Packard Auto-γ 500 counter (Hewlett Packard, Avondale, PA). Binding data were analyzed by the curve-fitting program GraphPad Prism (GraphPAD Software, San Diego, CA). Hill coefficients were determined from the slope of the log-logit curve. For saturation binding studies, [^{125}I]CYP concentrations ranging from 5 to 200 pM were used. Saturation curves were obtained by incubating cell membranes with increasing amounts of [^{125}I]CYP in the same buffer system used for the competition studies. To reduce interassay variation, binding assays were performed simultaneously with all constructs.

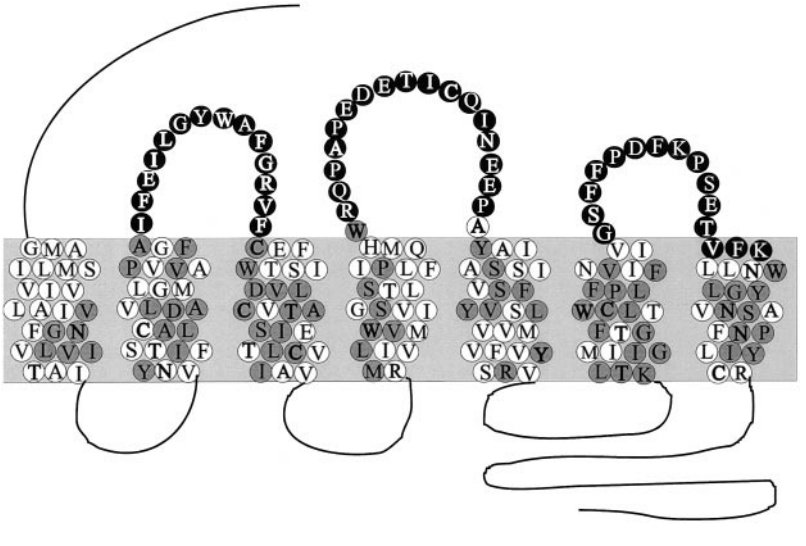

Fig. 1. Schematic of the chimeric hamster lung β_2-AR with corresponding replacements in the extracellular loops with rat α_{1a}-AR residues (in *black*). Residues in *gray*, amino acids in the β_2-AR which are identical to that of the α_{1a}-subtype; residues in *white*; unique to the β_2-AR.

Coupling analysis. Coupling analysis is based upon the conversion of a two-site into a one-site model upon the addition of GTPγS (0.1 mM) in the competition binding of agonists. The high affinity site is taken to be the fraction of receptors precoupled to the G protein, whereas the low affinity site is the uncoupled state. One-site versus two-site models were tested for best fit by the *F* test, and significance was determined for a p value of 0.05 or lower using the program GraphPad Prism. Two site model conforms to the equation

$$Y = \text{bottom} + (\text{top} - \text{bottom})\left[\frac{\text{fraction 1}}{1 + 10^{X - \log EC_{50_1}}} + \frac{1 - \text{fraction 1}}{1 + 10^{X - \log EC_{50_2}}}\right]$$

where Y is binding (specific) and X is the logarithm of the concentration of the unlabeled ligand. Top and bottom are the plateaus of the binding curve. Fraction 1 is the fraction of the receptors that have an affinity described by log EC_{50_1}. The remainder of the receptors have an affinity described by log EC_{50_2}.

cAMP assay. Accumulation of cAMP in WT and chimeric transfectants was measured using a commercially available cAMP assay system (Amersham) according to the directions supplied by the manufacturer. Cell extracts were derived from cultures in 60-mm dishes that were preincubated for 30 min with 5 mM theophylline and then for 30 min with both theophylline and increasing concentrations of agonist at 37°. Cells were lysed with 0.1 M HCl and diluted to obtain values within a standard curve supplied with the kit. Receptor density was determined by saturation binding on plates that were cotransfected in parallel studies to the signal transduction. All constructs were measured in the same assay to reduce interassay variations. Statistical significance ($p < 0.05$) in both binding and function were determined by a Student's t test or a one-way ANOVA followed by a Student-Newman-Keuls multiple comparison test.

Materials. Drugs were obtained from the following manufacturers: isoproterenol, epinephrine, WB4101, phentolamine, propranolol, albuterol, and alprenolol, Sigma (St. Louis, MO); ICI 118551, Research Biochemicals Inc. (Natick, MA); [^{125}I]CYP and cAMP kit were from Amersham Corp. Bio-Gel A-50m resin was from BioRad.

Results and Discussion

The adrenergic subtypes, although structurally similar in their transmembrane domains, have significant differences in their ligand-binding affinities for a number of synthetic agonists and antagonists. Determination of the critical amino acids responsible for these differences may assist in the design of better subtype-selective drugs. Previous work exploring these determinants in α_1-subtypes found that three adjacent residues in the putative second extracellular loop region near the fifth TM were fully responsible for the higher binding affinity (10–100-fold) of the α_1-antagonists, WB4101 and phentolamine, seen in the α_{1a}-subtype as compared with the α_{1b} subtype (Zhao *et al.*, 1996). The data indicated that α_1 antagonists may bind either nearer the surface of the receptor than the binding of agonists or that the antagonists may bind either partly or mostly in the extracellular loop regions. To address these possibilities, we constructed a series of β_2/α_{1a} chimeras in which we replaced the extracellular loop regions of the β_2-AR with the corresponding regions of the α_{1a}-AR. The regions replaced are shown in black in Fig. 1. Residues shown in gray represent amino acids in the β_2-AR that are identical to similar positions in the α_{1a}-AR. Transmembrane boundaries are tenative based upon no structural data. Given the extracellular changes and the high degree of homology in the transmembrane regions between the β_2-AR and the α_{1a}-AR, we rationalized that, if most of the antagonist binding determinants for α_1-ARs were localized in the extracellular loop regions, we could impart to the β_2-AR a higher binding affinity for α_1-antagonists via generation of these chimeras, representing a gain of function paradigm.

All of the chimeric constructs expressed functional receptors in transiently transfected COS-1 cells. [^{125}I]CYP labeled an apparently homogenous population of binding sites with similar affinities in membranes prepared from the chimeras or WT β_2-AR (Table 1). Binding of [^{125}I]CYP was statistically best fit to a one-site model. As shown in Fig. 2, replacement of individual or of all three extracellular loop regions did not change the affinity of either α_{1a}-antagonist, WB4101 or phentolamine, for the β_2-AR. This finding contradicts our hypothesis that our chimeric receptors would exhibit higher α_1-antagonist binding affinity than would the WT β_2-AR. However, the binding pocket of the β_2-AR is quite distinct from the α_1-AR, and there is precedent for even similar receptors (α_2-AR versus β_2-AR) showing distinct helical orientations and packing arrangements such that only functional receptors can be generated from certain chimeric combinations (Mizobe *et al.*, 1996). This would suggest that the adrenergic receptors are quite distinct from each other in the arrangement of the helices and ultimately, the binding pocket, even though they bind the endogenous ligands with similar affinity and have many amino acids in common. As evidence for this, mutagenesis studies have shown that the serines in TM 5 that are utilized in the binding of the catechol hydroxyls in the α_{1a}-AR are quite distinct in both usage and orientation from the β_2-AR paradigm (Hwa and Perez, 1996). Therefore, our chimeras may not have worked because the binding of phentolamine and WB4101 in the WT β_2-AR may be in a quite distinct orientation from the α_1-AR.

TABLE 1

Ligand binding profiles of WT β_2-AR and various chimeras

Competition binding studies were used to determine the IC_{50} (−Log) values (mean ± standard error) of adrenergic ligands, as described in Experimental Procedures, using [^{125}I]CYP as the radioligand and membranes prepared from COS-1 cells expressing WT β_2-AR or chimeras. Receptor densities (B_{max}) were determined on the same membranes from equilibrium saturation studies. Bold values indicate significant increases ($p < 0.05$–0.001) in affinity from the corresponding WT β_2-AR (one-way ANOVA followed by Student-Newman-Keuls multiple comparison test). Numbers in parentheses represent Hill coefficients. $n \geq 3$/each group. EX2, EX3, and EX2+3 are β_2-ARs with the corresponding EX replaced with the α_{1a}-AR sequence. EX1+2+3 is the β_2-AR with all three EX replacements.

	β_2-WT	EX2	EX3	EX2+3	EX1+2+3
β-AR Agonists					
Epinephrine	6.85 ± 0.1 (.6)	6.79 ± 0.23 (.6)	**7.88 ± 0.06 (.3)**	**7.75 ± 0.17 (.4)**	**7.94 ± 0.04 (.4)**
Isoproterenol	7.48 ± 0.1 (.6)	7.79 ± 0.1 (.6)	**8.53 ± 0.03 (.4)**	**8.65 ± 0.13 (.35)**	**8.61 ± 0.19 (.35)**
Albuterol	6.39 ± 0.1 (.5)			**7.22 ± 0.09 (.37)**	**7.62 ± 0.37 (.4)**
β-AR Antagonists					
ICI 118551	8.55 ± 0.19 (.7)			8.27 ± 0.27 (.6)	8.34 ± 0.2 (.6)
Alprenolol	9.15 ± 0.51 (.6)			9.30 ± 0.13 (.6)	9.05 ± 0.25 (.6)
B_{max} (pmol/mg)	0.140 ± 0.02	0.100 ± 0.01	0.050 ± 0.01	0.040 ± 0.01	0.050 ± 0.004
K_D (pM) [^{125}I]CYP	40.1 ± 4.9	64.7 ± 6.5	51.9 ± 6.7	36.9 ± 5.4	60.2 ± 8.1

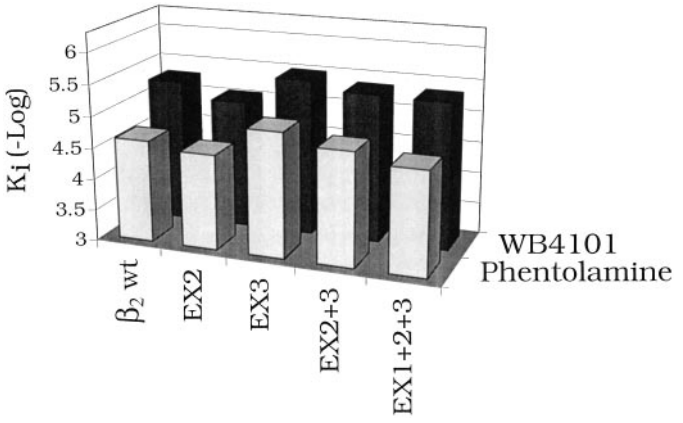

Fig. 2. Binding affinities of the β_2/α_{1a} chimeras for α_{1a}-AR selective antagonists. The designations of EX2, EX3, etc., are β_2-ARs in which the first, second, or third EX have been switched to corresponding to α_{1a}-residues either individually or in combination. Affinities were not significantly different from the WT β_2-AR control as determined by an ANOVA. Results are based on three or four experiments performed in duplicate.

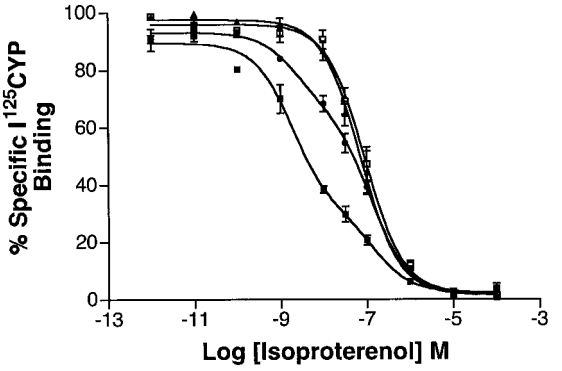

Fig. 3. Multiple binding site and GTP-shift analysis of EX3 and WT receptors. Competition curves were subjected to one-site versus two-site analysis using GraphPad Prism as explained in Experimental Procedures. The high affinity site was tested for G protein precoupling by the addition of GTPγS (0.1 mM) to the binding assay and measuring the conversion to the low affinity site. Data are shown in Table 2. ■, isoproterenol competition of EX3; ●, isoproterenol competition of WT β_2-AR. ▲, □, superimposed competition curves upon the addition of GTPγS for EX3 and WT, respectively. These assays were performed at equal receptor number of 50 or 54 ± 6.6 fmol/mg of membrane protein for either construct. $n \geq 3$ for each determination; shown are composite curves.

Even though our α_1-antagonist binding hypothesis seems incorrect, a routine assessment of these chimeras in their ligand binding properties revealed an unexpected phenotype. Table 1 indicates the pharmacological characteristics of the WT β_2-ARs and the chimeric ARs. Competition binding studies with various β_2-agonists and antagonists revealed higher IC_{50} values (10-fold) for agonists in some of the chimeras than the WT receptor with no changes in the antagonist values. The higher affinity for agonists was specific for the chimeras with changes in EX3 because EX3, EX2+3 and EX1+2+3 all displayed higher affinity, whereas EX2 alone showed no change in affinity. These results suggest that perhaps the EX3 replacement rendered this β_2-AR chimera constitutively active, because a higher binding affinity for agonists and not antagonists is a common characteristic for CAM receptors (Samma *et al.*, 1993).

However, higher agonist binding affinity is also seen in membrane preparations of β-ARs that partition into high and low affinity sites based upon G protein precoupling. Although high affinity sites are typically difficult to obtain in transiently transfected COS-1 cell membranes because of receptor overexpression in a limited number of cells and, thus, a disproportionate amount of receptors are in an uncoupled state, reduction of receptor density to the low femtomolar range can sometimes reveal mixed populations. To explore this possibility, a detailed analysis of isoproterenol binding with and without GTPγS was performed at low receptor number. As shown in Table 2 and Fig. 3, binding of isoproterenol was statistically best fit to a two-site model in both WT receptor and the EX3 chimera. To assure that receptor number and thus, the receptor/G protein ratio was similar in both preparations, the binding studies were performed under a similar receptor density. Because the EX3 chimera had a fixed expression under 100 fmol/mg of membrane protein that could not be altered significantly with larger amounts of DNA, we lowered the amount of the WT construct used in the transfection until a similar expression was achieved. Nevertheless, the fraction of high affinity sites in EX3 (63%) was twice that of the WT control (30%), and all of the high affinity sites were converted to low affinity sites with GTPγS (Fig. 3). This suggests that the agonist high binding affinity seen in EX3 was not an intrinsic property of the receptor but rather because of precoupling of the receptor to G protein(s). Although the G protein precoupling phenomenon in binding studies has never been directly and proportionately correlated to the formation of an active ternary complex, it is assumed to represent some relationship of the affinity of the receptor for the G protein in the presence of agonist. Given this, the results are consistent with the interpretation that the extracellular loop replacement altered G protein coupling in which EX3 formed a ternary complex much easier than WT control because of its higher affinity/coupling to the G protein.

If the rate of formation of an active ternary complex in EX3 was indeed greater than the WT control and not because of some artifact or alteration in receptor/G protein ratio in membrane preparations, we should observe concomitant increases in both the basal and agonist-dependent changes in signal transduction in whole cell studies. Therefore, basal cAMP production was measured in COS-1 cells transfected with the chimeric or WT receptor. As shown in Fig. 4, even though the WT and EX2 expresses more receptor, EX3 and EX1+2+3, displayed a significantly higher basal cAMP activity (70% over WT, $p < 0.001$), which is commensurate with the 100% increased fraction of high affinity sites in EX3. This constitutively active receptor is not fully active but represents a basal activity that is 20% of a fully activated WT receptor (Fig. 5). The constitutive activity of EX3 was re-

TABLE 2

Analysis of isoproterenol binding profiles

Competition curves were subjected to two-site analysis using the program GraphPad Prism. The equilibrium binding constants for high ($-pK_H$) and low ($-pK_L$) affinity sites were determined if a two-site model was significantly better ($p < 0.05$) than a one-site model. R_H is the fraction of receptors in the high affinity state. p is the significance for the two-site model. Receptor expression was 50–54 ± 6.6 fmol/mg of membrane protein for both WT and EX3 constructs. $n \geq 3$ for each determination.

	$-pK_H$	$-pK_L$	% R_H	p
WT β_2-AR	8.63 ± 0.14	6.74 ± 0.29	30.2	0.002
WT β_2-AR + GTPγS		7.03 ± 0.26	0	0.88
EX3	8.88 ± 0.2	6.75 ± 0.22	63.5	0.009
EX3 + GTPγS		7.08 ± 0.18	0	0.38

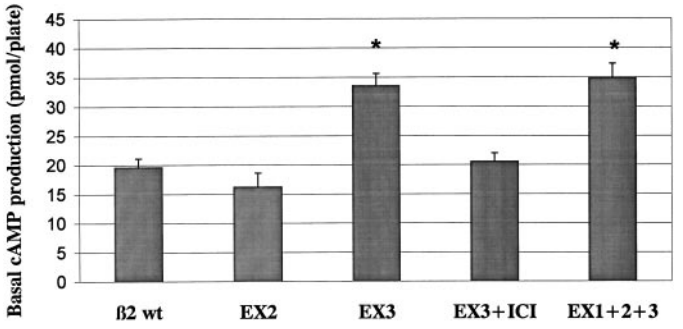

Fig. 4. Basal cAMP production in the absence of agonist by the β_2/α_{1a} chimeras. Expression levels of receptors were 98 ± 18, 75 ± 10, 49 ± 7, and 53 ± 5 fmol/mg of membrane protein for the WT β_2-AR, EX 2, EX3, and EX 1+2+3, respectively. *, significance ($p < 0.026$) from the WT β_2-AR control as determined by an ANOVA followed by the Student-Newman-Keuls multiple comparison test. ICI 118551 (*ICI*) was used at 10 μM to reverse the effects of the constitutive activity of EX3. Results are based upon five or six experiments performed in triplicate.

versed upon the addition of the inverse agonist, ICI 118551. Agonist-dependent changes in cAMP production is shown in Fig. 5. Even though EX3 expressed half the receptors of WT, the isoproterenol-stimulated EC_{50} of the cAMP response for EX3 was 4-fold lower (a significantly higher potency) than WT control (7.4×10^{-7} M versus 2.8×10^{-6} M, $p < 0.03$). Even though the degree of constitutive activity was not proportional to the amount of high affinity sites, both of these whole cell studies are consistent with the EX3 chimera being able to generate more active ternary complex than the WT control.

All in all, the data indicate that replacement of EX3 by corresponding α_{1a}-AR residues generates a constitutively active receptor, because signal transduction can occur without the presence of agonist. However, the mechanism behind this constitutive activity is distinct from the mechanism described by all other CAM models previously characterized in adrenergic receptors (Samma *et al.*, 1993; Perez *et al.*, 1996; Hwa *et al.*, 1996, 1997; Porter *et al.*, 1996). Earlier models of constitutive activity have shown that the high affinity binding of agonists is independent of G protein coupling either as assessed by no changes in agonist binding with GTP analogs or in receptor solubilization studies (Samma *et al.*, 1993), where no G protein can interact. CAMs based on these earlier studies, are thought to cause intrinsic changes to the receptor, a G protein-independent conformational change that causes an increase in the J or isomerization constant of the receptor in isomerizing from R to R* (Fig. 6A). In addition, CAMs were shown to have little effect on the M constant, a receptor to G protein affinity constant in which an increased value would result in an enhanced coupling of the receptor to the G protein and formation of an active R*G (Fig. 6A), although this has not been empirically tested with purified components. Although computer simulations of a constitutively active β_2-AR and the basis for the revision of the ternary complex model (1) (Fig. 6A) indicated a minor contribution was because of altered G protein coupling (the M constant), this was necessary to account for the full extent of the increase in basal signal transduction which is mostly because of changes in J. This revision of the ternary complex model was necessary to account for the existence of an efficacy-related change in affinity for the uncoupled state (G protein independence) of the mutant receptor. In contrast, the EX3 results would suggest that the M constant and not the J constant is being altered in this model because its constitutive activity and high affinity state was dependent on the G protein. However, the revised TCM also makes a large assumption that only the R* form is able to bind and activate the G protein, so that HR*G is the only possible ternary

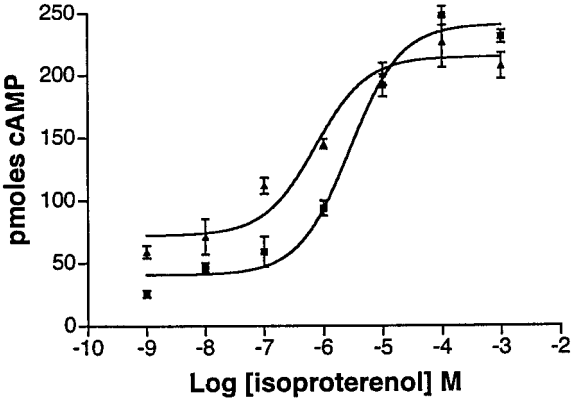

Fig. 5. The potency of isoproterenol to stimulate cAMP production in WT versus EX3 chimera. COS-1 cells expressing the WT (■) or EX3 chimera (▲) were stimulated with increasing concentrations of isoproterenol to generate cAMP as described in Experimental Procedures. Receptor expression was 202 ± 41 and 92 ± 32 fmol/mg of membrane protein for the WT or EX3 chimera, respectively. EC_{50} for the EX3 chimera ($7.4 \pm 1.5 \times 10^{-7}$ M) was achieved at a significantly lower isoproterenol concentration ($p < 0.036$) when compared with the WT β_2-AR control ($2.8 \pm 1.2 \times 10^{-6}$ M). Both transfected cell lines gave similar increases in the forskolin-stimulated cAMP response. EC_{50} and receptor density values are presented as the mean ± standard error for three to five experiments performed in triplicate.

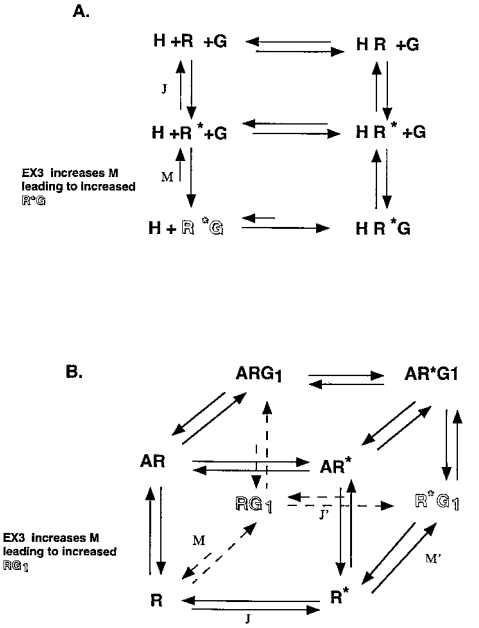

Fig. 6. Ternary complex models of receptor activation. A, The revised ternary complex model as described by Samma *et al.* (1993). R undergoes an allosteric isomerization with a rate constant J, which leads to the formation of an R* intermediate. R* can then bind and activate G protein leading to the formation of an active species, R*G, governed by the rate constant, M. H, hormone; *R*, receptor; *G*, G protein; *B*, the cubic ternary complex model as described by Kenakin (1995). R can isomerize to R* with a rate constant J; however, R can also bind G protein with a rate constant M leading to the formation of the inactive species, RG_1. RG_1 can then go on to form the active species, AR^*G_1 or R^*G_1 through different rate constants. *A*, agonist; *R*, receptor; G_1, one G protein. For clarity, the G_2 (or second G protein) pathway has been eliminated.

complex formed. Alternately, according to the cubic ternary complex model of Kenakin (1995) (Fig. 6B), the EX3 chimera could have altered equilibrium because of an increased M constant but the commensurate increase in the inactive species, RG_1, would ultimately effect the equilibrium leading to a greater amount of the active species, R^*G_1 or AR^*G_1. Although our data here cannot distinguish between these two models, the results suggest that the EX3 constitutive activity was because of an altered affinity/coupling for the G protein, which ultimately through mass action influences the amount of active complex that is formed. This is distinct from other CAMs that increase active species directly without alterations in G protein coupling.

Because the location of the replacement in the EX3 chimera is in the extracellular environment, it is not possible to propose a direct interaction of these residues in altering the affinity of the receptor to the G protein. There is precedent, however, in soluble proteins that loop regions can influence the arrangements and packing of α-helices (Munoz *et al.*, 1995). Our observations are most likely indirectly attributable to helical changes/packing in TM 6 and/or 7, which have been implicated as important movements in the activation process of rhodopsin (Altenbach *et al.*, 1996). These changes could effect the conformation of the third intracellular loop, which is implicated in the binding and activation of G proteins. Nevertheless, it is interesting to note that the constitutive activity in EX3 was independent of any subsequent changes in EX1 and/or EX2, suggesting that the conformation adopted by EX3 was fixed and nonreversible as though a constraining factor was broken. The implications of the current study reinforce an emerging concept that the entire structure of G protein-coupled receptors have evolved to constrain the receptor in the inactive state. Altered structure through mutagenesis of the intracellular loops (Samma *et al.*, 1993), transmembrane domains (Perez *et al.*, 1996; Hwa *et al.*, 1996, 1997; Porter *et al.*, 1996), and now extracellular loops have all led to constitutive activity, although the mechanism behind these changes may be different.

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Send reprint requests to: Dr. Dianne M. Perez, Department of Molecular Cardiology, FF3-01, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, Ohio 44195. E-mail: perezd@cesmtp.ccf.org