Chimeras of $\alpha_1$-Adrenergic Receptor Subtypes Identify Critical Residues That Modulate Active State Isomerization*

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We have identified previously two amino acids, one in each of the fifth and sixth transmembrane segments of both the $\alpha_1_a$-adrenergic receptor and the $\alpha_1_b$-adrenergic receptor (AR), that account almost entirely for the selectivity of agonist binding by these receptor subtypes (Hwa, J., Graham, R. M., and Perez, D. M. (1995) J. Biol. Chem. 270, 23189–23195). Thus reversal of these two residues, from those found in the native receptor of one subtype to those in the other subtype, produces complementary changes in subtype selectivity of agonist binding. Here we show that mutating only one of these residues in either the $\alpha_1_b$-AR or the $\alpha_1_a$-AR to the corresponding residue in the other subtype (Ala$^{293} \rightarrow$ Val for the $\alpha_1_b$-Met$^{292} \rightarrow$ Leu for the $\alpha_1_a$-AR) results in chimeras that are constitutively active for signaling by both the phospholipase C and phospholipase A$_2$ pathways. This is evident by an increased affinity for agonists, increased basal phospholipase C and phospholipase A$_2$ activation, and increased agonist potency. Although mutation of the other residue involved in agonist binding selectivity, to the corresponding residue in the other subtype (Leu$^{214} \rightarrow$ Met for the $\alpha_1_a$-AR; Val$^{185} \rightarrow$ Ala for the $\alpha_1_b$-AR) does not alter receptor binding or signaling, per se, when combined with the corresponding constitutively activating mutations, the resulting chimeras, Ala$^{293} \rightarrow$ Val/Leu$^{214} \rightarrow$ Met ( $\alpha_1_b$-AR) and Val$^{185} \rightarrow$ Ala/Met$^{292} \rightarrow$ Leu ( $\alpha_1_a$-AR), display wild type ligand binding and signaling. A simple interpretation of these results is that the $\alpha_1_a$- and $\alpha_1_b$-ARs possess residues that critically modulate isomerization from the basal state, R, to the active state R*, and that the native receptor structures have evolved to select residues that repress active state isomerization. It is likely that the residues identified here modulate important interhelical interactions between the fifth and sixth transmembrane segments that inhibit or promote receptor signaling.

$\alpha_1$-Adrenergic receptors are members of the G-protein-coupled receptor superfamily. All three cloned subtypes ($\alpha_1_a$, $\alpha_1_b$, and $\alpha_1_d$) activate multiple signal transduction pathways via distinct G-proteins and effectors, including phospholipase C (PLC) and A$_2$ (PLA$_2$) (1, 2).

Recently a number of point mutations have been described in both the $\alpha_1_a$-AR and other adrenergic receptors that result in partial activation in the absence of agonist stimulation (3, 4). Such mutations are not unique to the adrenergic receptor superfamily, since constitutively active somatic mutations of the human thyrotropin receptor that result in adenomas and hyperthyroidism have also been identified (5). In addition, mutant hyperfunctioning luteinizing hormone receptors have been reported that lead to precocious puberty in young males (6). In general, these mutations are characterized by increased agonist binding, increased agonist independent receptor activation, and increased agonist potency (EC$_{50}$). The increased agonist binding is independent of G-protein binding and thus is an intrinsic property of the receptor. Additionally, constitutive activity of these mutants can be reversed by some antagonists. Based on these findings, as well as the observation that over-expression of wild type receptors can also initiate biochemical responses in the absence of agonist, it has been proposed that receptors spontaneously isomerize between a basal state, R, and an active state, R*. Furthermore, agonists bind with higher affinities to the active state and “trap” the receptor in the R* conformation.

With regard to the hamster $\alpha_{1b}$-AR, two noteworthy mutations have been identified. Substitution of Ala$^{393}$ in the third intracellular loop to any of the other amino acids results in varying degrees of constitutive activity (3). In addition, in the third transmembrane domain, replacement of the natural cysteine residue, Cys$^{128}$, by phenylalanine also results in constitutive activity (4). However, by contrast with the Ala$^{393}$ mutants, the increased basal effector activation and increased agonist potency observed with the Cys$^{128} \rightarrow$ Phe mutation are pathway-specific, since they are confined almost entirely to the PLC pathway. Here we show that when one of two residues in either the $\alpha_{1a}$- or the $\alpha_{1b}$-ARs, which we have shown previously are involved in determining subtype selectivity for agonists (7), is mutated to the corresponding residue in the other subtype (Ala$^{293} \rightarrow$ Val, $\alpha_{1b}$-AR; Met$^{292} \rightarrow$ Leu,$^2 \alpha_{1a}$-AR), the resulting chimeras are constitutively active for both the PLC and PLA$_2$ pathways. Mutations in Val$^{185}$ in the $\alpha_{1a}$-AR (the residue corresponding to Ala$^{293}$ in the $\alpha_{1b}$-AR) or Leu$^{314}$ in the $\alpha_{1b}$-AR (the residue corresponding to Met$^{292}$ in the $\alpha_{1a}$-AR) when replaced by the corresponding residue in the other subtype, are not constitutively active. However, when combined with the constitutively activating mutants, the resulting double mutants, $^2$...
**α1a-Adrenergic Receptor Constitutive Activity**

Ala\(^{204}\) → Val and Val\(^{185}\) → Met and Val\(^{185}\) → Ala\(^{204}\) → Leu, show a restoration of native receptor signaling. This paper documents that active state isomerization of receptors can be modulated by the composition of specific amino acids in adjacent transmembrane domains.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**

The construct used was the hamster \(\alpha_{1a}\)-AR cDNA and rat \(\alpha_{1a}\)-AR cDNA described previously (7). Site-directed mutagenesis was performed as described previously (7), utilizing the oligonucleotide-mediated double primer M13 method (8). Mutations were confirmed by DNA sequencing.

**Transfection of COS-1 Cells**

COS-1 cells (ATCC) were transfected with the various constructs as described previously (7) using the DEAE-dextran method. Initial transfections for ligand binding analysis were performed with 5 µg of DNA/ml of transfection solution. To achieve equal receptor numbers for the functional assays, the Ala\(^{204}\) → Val construct was reduced to 2 µg of DNA/ml of transfection solution while the wild type receptor was reduced to 1 µg of DNA/ml of transfection solution. Transfection efficiencies for the DEAE-dextran method ranges from 10 to 20%

**Ligand Binding**

The ligand binding characteristics of the expressed receptors were determined in a series of radioligand binding studies using \([^{125}\text{I}]\text{HEAT}\), an \(\alpha_{1a}\)-specific antagonist (9). Membranes were prepared as described previously (9). For competition studies, the procedure involved duplicate tubes containing 200 pm \([^{125}\text{I}]\text{HEAT}\), HEM buffer (20 mM HEPES, pH 7.5, 1.4 mM EGTA, 12.5 mM MgCl\(_2\)), and competing ligand (at 10 or more different dilutions). Nonspecific binding was determined by the addition of phospholipids (10\(^{-4}\) M). For saturation binding studies, 80–1600 pm \([^{125}\text{I}]\text{HEAT}\) was used. After 1 h of incubation at room temperature, the reactions were stopped by the addition of ice-cold HEM buffer and were filtered onto Whatman GF/C glass filters with a Brandel Cell Harvester. The filters were washed five times with ice-cold HEM. They were then analyzed for bound radioactivity using a Packard Auto-gamma 500 Counter.

Data were then analyzed with the interactive program LIGAND. IC\(_{50}\) values were expressed as the mean ± standard error of the mean. ANOVA and Student’s t-test were used to determine significant differences (p < 0.05). To detect small but significant differences, sets of mutations along with the two control wild type receptors were evaluated simultaneously (i.e. in the same assay).

**Inositol Phosphate Determination**

\(^{3}H\) Radio-receptor Assay (DuPont)—We used this technique to accurately measure the total basal production of IP\(_3\). In brief it involved washing the cells in Hank’s balanced salt solution, followed by a 30-min incubation in 10 mM LiCl. Trichloroacetic acid (100%) was then used to disrupt the cells and the cell debris removed with ultracentrifugation. The trichloroacetic acid was removed by 1,1,2-trichloro-1,2,2-trifluoro-ethanethiin-4-iodoacetamine (3:1) extraction. The resultant supernatant was applied to 1-ml packed AG1-X8 (Bio-Rad) columns (100–200-mesh, formate form). The column was washed with 16 ml of 0.1 M formic acid, and total inositol phosphates were eluted with 1 M ammonium formate, 0.1 M formic acid in 2-ml fractions. Scintillate was added and the fractions counted. Functional assays and equilibrium binding studies (to determine receptor densities) were performed on sequential fractions in which equal quantities of DNA were used.

**Arachidonic Acid Assay**

This was performed according to a previously published protocol (1). Briefly, 24 h prior to assay the transiently transfected cells were washed once in serum-free DMEM followed by the addition of \([^{3}H]\text{arachidonic acid (1 µCi/ml)} and serum-free DMEM. To inhibit receptor signaling, phentolamine, an \(\alpha_{1a}\)-AR-specific antagonist, was added to a final concentration of 100 µM. On the day of assay, the cells were washed twice with serum-free DMEM and incubated for 30 min. Phenolone, a dual cyclooxygenase and lipooxygenase inhibitor, was added to a final concentration of 100 µM for 30 min. Agonists were added, and after another 30 min, the cells were removed and the c1 medium was removed. The fluid and debris were scraped into tubes, and 1 ml of MEOH:CHCl\(_3\):HCl (40:20:0.5; v/v/v) was added. The tubes were vortexed and centrifuged to remove debris. The supernatant, which contained released arachidonic acid, was added to 20 ml of scintillation mixture, dark-adapted, and counted for radioactivity. The counts indicated the relative amount of arachidonic acid released. Prior experiments with thin-layer chromatography have indicated the identity of the radioactivity as arachidonic acid (1).

**Materials**

Drugs were obtained from the following manufacturers. \((-\)Epi-nephrine, \((-\)norepinephrine, oxymetazoline, methoxamine, \(-\)phentolamine, and phentolamine were from Sigma. Cirazoline was a gift from Pfizer. \([^{125}\text{I}]\text{HEAT}, \[^{125}\text{I}]\text{inositol, \[^{125}\text{I}]\text{arachidonic acid, and the \[^{125}\text{I}]\text{IP}_{3} radioiodide kit were from DuPont NEN. AG1-X8 was from Bio-Rad.**

**RESULTS**

**Pharmacological Characterization of Ala\(^{204}\) → Val and Leu\(^{184}\) → Met (\(\alpha_{1b}\)-AR)—Ala\(^{204}\) → Val and Leu\(^{184}\) → Met in the \(\alpha_{1b}\)-AR are located in the fifth and sixth transmembrane domains, respectively, and are believed to face the ligand binding pocket (Fig. 1) (7, 10). We have shown previously that these residues are critical in defining an \(\alpha_{1b}\)-AR agonist-selective pharmacology. The double mutant, Ala\(^{204}\) → Val/Leu\(^{184}\) → Met, has an \(\alpha_{1a}\)-AR agonist pharmacology (7). For the purpose of clarity, some of these data are repeated in Table I. Full pharmacological characterization of these mutants are described in detail elsewhere (7). As shown in Table I, the single mutant Ala\(^{204}\) → Val exhibits an increased binding affinity (3–10-fold) for the catecholamines epinephrine (p < 0.001) and norepinephrine (p < 0.001). This mutant also has a 10-fold increase in affinity for oxymetazoline (p < 0.001) as well as for other imidazoline agonists, a phenomenon not seen with two other constitutively active mutations of this receptor (4). This indicates that the enhanced affinity is the result of a shift in the pharmacological specificity of the ligand binding pocket due specifically to the Ala\(^{204}\) → Val mutation. This increased binding affinity does not arise from an effect due to enhanced G-protein coupling, since pertussis toxin pretreatment (1 µg/ml) or Gpp(NH)p, which uncouples receptor/G-protein interactions, did not alter the increase in agonist binding affinity (data not shown). There was no significant change in antagonist binding (data not shown), including the affinity of Ala\(^{204}\) → Val for \([^{125}\text{I}]\text{HEAT}\) (K\(_{d}\) 61 ± 10 pm), as compared to the published affinity of the wild type \(\alpha_{1b}\)-AR (K\(_{d}\) 78 pm) (11).

Leu\(^{184}\) → Met alone exhibited no significant increase in the binding of either the nonselective agonists, \((-\)epinephrine and \((-\)norepinephrine (Table I), or antagonists (data not shown). There was, however, an increased binding affinity for oxymetazoline and methoxamine, two \(\alpha_{1b}\)-selective agonists, as described previously (7). When Leu\(^{184}\) → Met was combined with Ala\(^{204}\) → Val in the same \(\alpha_{1b}\)-AR construct, there was an overall decrease in binding affinity for nonselective agonists as compared to the Ala\(^{204}\) → Val mutant alone. The additional mutation of Leu\(^{184}\) to methionine, therefore, had an inhibitory effect on the binding affinity for the two natural ligands. This
same trend was not apparent for the synthetic subtype-selective agonists.

Equilibrium binding studies revealed expression levels (3.0–3.9 pmol/mg protein, respectively) for both the Ala204 → Val and the Leu314 → Met single mutants which were similar to that observed with the wild type α2a-AR (Table I). When combined, the expression level of the double mutant (Ala204 → Val/Leu314 → Met) dropped to 1.2 pmol/mg protein, a value not dissimilar to that observed for the wild type α2a-AR (1.0 pmol/mg protein).

Inositol Phosphate Production—The ability of each construct to activate PLC was determined by the two techniques described under “Experimental Procedures.” To measure total basal levels of IP₃, a [³H]IP₃ radio-receptor assay (DuPont) was used on the transfected COS-1 cells. From this we were able to accurately measure total IP₃ production by the receptors in the absence of agonist. The Ala204 → Val mutant showed almost a 3-fold increase in basal activity over that of the wild type α1b-AR (p < 0.05) (Fig. 2A). As indicated above, our binding data indicated that the wild type α2a-AR, and the single mutants, Ala204 → Val and Leu314 → Met, are expressed more abundantly than the wild type α3a-AR (Table I). Therefore, to eliminate the possibility that any increase in activity may be related to receptor density alone, we expressed each construct at multiple levels: 0.6, 2.8, and 3.0 pmol/mg protein for the wild type α2a-AR and 0.6, 2.0, and 3.9 pmol/mg protein for the Ala204 → Val mutant. This was accomplished by changing the amount of cDNA transfected. This indicated that the increased basal activity observed with Ala204 → Val is directly related to receptor density. Graphs of basal activity versus receptor number for the mutants produced slopes that were 3 times greater than the wild type receptor (Fig. 2C), consistent with the 3-fold increased basal activity of Ala204 → Val. In the presence of the antagonist, phentolamine (100 µM) (added 24 h prior to assay), there was no significant difference (p = 0.05) in IP₃ production (Fig. 4A), indicating negative agonist activity.

To determine agonist potency for the PLC pathway, total inositol phosphates were determined by adding [³H]inositol 24 h before the assay and then measuring epinephrine-stimulated inositol phosphate release. The Ala204 → Val mutation showed a rightward shift in the (–)epinephrine concentration-response curve, as compared to the wild type α1b-AR or the α3a-AR. The EC₅₀ (concentration of agonist producing 50% of maximal activation) values ± S.E. were 11 ± 1 nM, 38 ± 4 nM, and 77 ± 8 nM, respectively (Fig. 5A). This change correlated with the −3-fold decrease in the Kᵢ value for epinephrine and norepinephrine with this mutant. Maximum (–)epinephrine-stimulated inositol phosphate turnover by the Ala204 → Val mutant was not different from that of the wild type receptor (Fig. 6C).

Arachidonic Acid Production—The ability of each construct to activate the PLA₂ pathway was determined by prelabeling the transfected COS-1 cells with [³H]arachidonic acid (1 µCi/ml) for 16–24 h and measuring agonist-stimulated [³H]arachidonic acid release. As shown in Fig. 3A, with the Ala204 → Val mutation, basal arachidonic acid release was increased above that observed with the wild type α1b-AR. However, there was no significant difference in arachidonic acid release between the wild type α1b-AR, and either the wild type α3a-AR or the Leu314 → Met mutation. As with IP₃ production, to eliminate the possibility that any increase in activity may be related to receptor density alone, we expressed each construct at multiple levels of cDNA transfected.

**Table I**

| Phenethylamines       | α1b-AR | α2a-AR | L314M | V185A/M292L | V185A | M292L |
|-----------------------|--------|--------|-------|-------------|--------|-------|
| (–)-Epinephrine       | 5.29 ± 0.04 | 5.77 ± 0.03** | 5.50 ± 0.04 | 5.43 ± 0.04 | 5.43 ± 0.05 | 5.29 ± 0.03 | 6.09 ± 0.01*** | 5.35 ± 0.08 |
| (–)-Norepinephrine    | 5.25 ± 0.08 | 5.76 ± 0.03** | 5.43 ± 0.06 | 5.42 ± 0.06 | 5.41 ± 0.05 | 5.02 ± 0.07 | 5.92 ± 0.03*** | 5.12 ± 0.10 |
| Phenylephrine         | 4.69 ± 0.06 | 5.55 ± 0.05** | 4.81 ± 0.05 | 4.90 ± 0.05 | 4.85 ± 0.05 | 4.81 ± 0.09 | 5.58 ± 0.03*** | 4.96 ± 0.04 |
| Methoxamine           | 3.16 ± 0.06 | 3.96 ± 0.06** | 3.76 ± 0.05** | 4.57 ± 0.05** | 3.79 ± 0.01** | 3.76 ± 0.08** | 4.34 ± 0.03 | 4.43 ± 0.12 |
| Imidazolines          | 6.05 ± 0.05 | 6.90 ± 0.04*** | 6.34 ± 0.03* | 7.27 ± 0.06** | 5.91 ± 0.08*** | 5.88 ± 0.06** | 7.03 ± 0.06** | 7.34 ± 0.10 |
| Cirazoline            | 5.64 ± 0.11 | 6.10 ± 0.09*** | 6.20 ± 0.02** | 6.49 ± 0.04*** | 5.99 ± 0.04** | 6.14 ± 0.01 | 6.57 ± 0.03 | 6.38 ± 0.06 |
| Bₘax (pmol/mg protein)| 3.00    | 3.90    | 3.03   | 1.22        | 1.17    | 1.66   | 1.26    | 1.01     |

*P*-values indicate significant differences from the α1b-AR (*, p < 0.05; **, p < 0.01; ***p < 0.001) or from the α1a-AR (***, p < 0.001). These data have been taken from previously published studies (7).
density to basal IP$_3$ release. Wild type (WT) expression was determined as described above. In this experiment only a few levels: 0.6, 2.8, and 3.0 pmol/mg protein for the wild type Ala204Val mutant. This indicated that the increased basal activity observed with Ala204Val is directly related to receptor density, graphs of basal activity versus receptor number for the wild type receptor was 0.04 pmol of IP$_3$/fmol of receptor, while that of the Ala204Val mutant was 0.12 pmol of IP$_3$/fmol of receptor. Mutants giving a slope 4 times greater than the wild type (WTa) (*, p < 0.05). Panel B, relationship of receptor density to basal arachidonic acid release. Wild type (**) or the Ala204Val mutant (**) were expressed in COS-1 cells at different receptor densities by varying the amount of cDNA used in each transfection. The slope of the wild type receptor was 5.7 cpm/fmol receptor, while that of the Ala204Val was 23.5 cpm/fmol receptor.

levels: 0.6, 2.8, and 3.0 pmol/mg protein for the wild type $\alpha_{1b}$-AR and 0.6, 2.0, and 3.9 pmol/mg protein for the Ala204Val. When this mutant, the EC$_{50}$ was decreased by approximately 10-fold compared to the wild type as outlined under "Experimental Procedures." Values shown are the mean ± S.E., for 10–20 separate 60-mm plates (one measurement per plate) from four separate transfections, each containing an internal control of wild type receptor and mock transfection. Mean receptor expression levels of the various constructs were determined from equilibrium binding studies and were 0.6 pmol/mg protein for the $\alpha_{1b}$-AR (WTb), 0.63 pmol/mg protein for the Ala204Val, 3.03 pmol/mg protein for the Leu314Met (L314M), and 1.22 pmol/mg protein for the combination Ala204Val/Leu314Met (A204V/L314M). From each plate, 4 ml of cytosolic supernatant from a total of 1 ml (per plate) was used to calculate total IP$_3$ production. This represented the background counts. Only the Ala204Val was the only construct that resulted in a basal IP$_3$ release that was statistically different from the wild type $\alpha_{1b}$-AR (WTb) (*, p < 0.05). Panel B, relationship of receptor density to basal arachidonic acid release. Wild type (**), or the Ala204Val (**), or the A204V/L314M were expressed in COS-1 cells at different receptor densities by varying the amount of cDNA used in each transfection. The slope of the wild type receptor was 5.7 cpm/fmol receptor, while that of the Ala204Val was 23.5 cpm/fmol receptor.

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Met292 to Leu in the type receptors for both IP and arachidonic acid production. Values for epinephrine-stimulated IP and arachidonic acid release (Fig. 5, C and D). Likewise, maximal IP$_3$ and AA stimulation by (-)-epinephrine with the double mutants did not significantly change from that observed with wild type receptors (Fig. 6, C and D).

**DISCUSSION**

Like other G-protein-coupled receptors, the molecular mechanism involved in $\alpha_1$-AR activation remains unclear. Some insights into receptor activation have come recently from the discovery of mutations that constitutively activate G-protein-coupled receptors. These studies suggest that the native receptors spontaneously isomerize between a basal (R) and activated (R*) conformation, with only the R* conformation being able to productively interact with the receptor-coupled G-protein and, thus, to result in effector activation. Furthermore, agonists bind to the R* state with higher affinity than to the R state and "trap" the R* conformation. For wild type receptors, the equilibrium between the R and R* states markedly favors the basal conformation, and, in the absence of agonist, effector activation is minimal, unless the receptors are highly overexpressed. With marked overexpression, the number of receptor molecules in the R* state increases sufficiently to allow effector activation in the absence of agonist, even though the ratio of molecules in the R versus R* state is unchanged. By contrast to the wild type receptors, with mutations that constitutively activate the receptor, the energetic requirements for spontaneous isomerization from the R to R* are reduced and thus at any instance more receptors are in the R* conformation. This allows effector activation in the absence of agonist and explains the other hallmarks of constitutive activity, i.e. an increase in both agonist binding and in agonist potency.

In this paper we have characterized novel $\alpha_{1a}$ and $\alpha_{1b}$ chimeras that involve residues previously shown to be major determinants of the selectivity of these subtypes for agonist binding. From our earlier experiments (7), we demonstrated that one of these chimeras, which involves a change of Ala204 in the $\alpha_{1b}$-AR to the equivalent residue in the $\alpha_{1a}$-AR (Val185), resulted in a mutant $\alpha_{1a}$-receptor that recognized a number of agonists including the natural ligands, (-)-epinephrine and (-)-norepinephrine, with higher affinity (Table I). Because both the wild type $\alpha_{1a}$ and $\alpha_{1b}$ARs bind both (-)-epinephrine and (-)-norepinephrine with similar affinity, we questioned whether the higher agonist affinity of the Ala204 $\rightarrow$ Val mutant signified that it was constitutively active. The results show that in addition to increased agonist binding, this mutant displayed all the properties of a constitutively activated receptor, including agonist-independent effector activation and increased agonist potency. What was also intriguing was that when this Ala204 $\rightarrow$ Val mutation was combined with a change in Leu314 (the other residue identified previously to be involved in subtype selectivity for agonists) to methionine (the equivalent residue in the $\alpha_{1a}$-AR), all three parameters of constitutive activity were suppressed. Thus, the phenotype of the double mutant was indistinguishable from the wild type $\alpha_{1b}$-AR, except for a reversal of its agonist-binding profile to that of the $\alpha_{1b}$-AR.

To confirm that this phenomenon was not merely serendipitous or unique to the $\alpha_{1b}$-AR structure, we reversed the mutations in the $\alpha_{1a}$-AR. Characterization of these chimeras revealed analogous but complementary effects to those observed with the $\alpha_{1b}$ chimeras. Mutation at Met292 to leucine (the...
equivalent residue in the α1b-AR) resulted in constitutive activity. Mutation of Val185 to alanine (the reverse of the Ala204 → Val mutation in the α1a-AR) did not change receptor binding or signaling from that observed with the wild type α1a-AR. However, when combined with the Met292 → Leu mutation all parameters of constitutive activity were suppressed, so that binding of (−)-epinephrine and (−)-norepinephrine, and signaling by the double mutant did not differ from the wild type α1a-AR.

To further characterize the constitutively active α1 chimeras, we evaluated antagonist binding and the effects of antagonists on basal effector activation. Phentolamine suppressed basal effector activation by both the α1a-AR constitutively active mutant, Ala204 → Val, and the α1b-AR constitutively active mutant, Met292 → Leu, indicating that phentolamine is a negative agonist. However, there was no change in the binding affinity of the mutants for phentolamine or other α1-agonists, including the α1a-selective compounds, 5-methylurapidil and WB4101. One would predict that these constitutively active mutants would recognize these compounds with lower affinity, since fewer receptor molecules would be in the R (basal) conformation that binds negative agonists with higher affinity than the R* conformation. Failure to detect a change in affinity, however, can be explained by the fact that the Ala204 → Val (α1b-AR) and Met292 → Leu (α1b-AR) mutations only result in partial activation. Thus, with these mutations the proportion of molecules in the R* state is presumably not large enough to detect a change in affinity. In support of this notion, combination of the Ala204 → Val mutation with two previously defined constitutively active mutations, Cys128 → Phe and Ala293 → Glu (4), resulted in not only increased constitutive activity, compared to either single mutation alone, but also a lower affinity (−10–20-fold) for a number of antagonists including phentolamine.3

Based on these findings, the question arises as to the mechanism(s) involved in the altered binding and signaling properties of these α1 chimeras. Differences based simply on alterations in agonist/receptor contacts due to altered properties of the substituting residues, in terms of hydrophobicity or size, as proposed previously to explain the changes in agonist binding alone(7), cannot simply explain the increased agonist-independ-
Constitutive Activity

Due to perturbations in the helical structure of the involved transmembrane segments. The amino acid composition of an α-helix, for example, has been found to be critical for determining conformation (12–15). Also, changing the amino acids in an α-helix can perturb interhelical interactions (16). In this regard, the complementarity of the binding and signaling effects observed with the chimeras indicates there are critical interactions between the fifth and the sixth transmembrane segments. Thus the residues identified here may influence helical conformation, either by direct intrahelical effects or indirectly by an effect at the level of the side-chain packing between adjacent helices.

Although structural resolution at the atomic level will be required eventually to discern effects on helical conformation, based on the following considerations, we propose a model involving predominant effects on interhelical packing, which accounts for the phenotypes observed with the chimeras, and provide insights into the mechanism(s) of G-protein-coupled receptor activation. First, it is of interest that the Ala204 → Val constitutively activating mutation, as well as a previously characterized constitutively activating α2β AR mutation, Cys128 → Phe, involve transmembrane residues that are located approximately one helical turn above or below residues putatively involved in forming critical interactions with agonists. Thus, in the fifth transmembrane domain the Ala204 is one helical turn above Ser207, which hydrogen-bonds with the catechol hydroxyl of phenethylamines; and in the third transmembrane domain Cys128 is one helical turn below Asp125, which forms a salt bridge with the protonated amine of agonists. This suggests that these mutations alter the conformation of the third and fifth transmembrane segments, or the relative positioning of the helices, leading to an enhanced propensity for the receptor to isomerize spontaneously to the R* state, as evidenced by increased basal signal transduction, an agonist-independent manifestation of constitutive activity. However, this conformational change may also facilitate the bonding of critical residues such as Ser207 and Asp125 with agonist, thus leading to agonist-dependent manifestations of constitutive activity such as high affinity binding and increased potency. Second, the constitutive activity of the Met292 → Leu α1a AR chimera, suggests that a conformational change in the sixth transmembrane segment may also be involved in receptor signaling. However, this may be indirect through an interaction with the fifth helical segment, which then also influences agonist bonding with Ser207, since mutation of Val185 in the fifth transmembrane segment to Ala relieves the constitutive activity of Met292 → Leu.

Based on these considerations, we propose that the constitutive activity of the Ala204 → Val is not due to the loss of alanine, which in the native receptor structure may be postulated to act as a repressor of active state isomerization. Rather, the valine, either because of its bulk or β-substituent, prevents normal side-chain packing between the fifth and the sixth helical segments. As a result there is a conformational distortion of the fifth helical segments that mimics the active state conformation and allows enhanced interactions between Ser207 and the agonist (Fig. 7). With the additional substitution of Leu314 by a methionine residue, this conformational distortion is relieved and the receptor is no longer constitutively active. Similarly, with the native α1a AR there is normal packing between the fifth and the sixth helical segments, despite the presence of the wild type valine at position 185, since the native methionine at position 292 allows the valine to be accommodated. However, with a leucine at position 292, as occurs in the Met292 → Leu mutation, valine at position 185 now induces constitutive activation, since normal interhelical packing is prevented. Finally,

![Fig. 6. Dose-response curves of IP and arachidonic acid release for the single and double α1a-mutants using (-)-epinephrine as the agonist. Panel A, dose-response curve for IP production using varying concentrations of (-)-epinephrine. Each data point represents the mean ± S.E. derived from eight separate experiments. With the Met292 → Leu mutation, there was a shift to the left in the dose-response curve. However, with the double mutation, Met292 → Leu/Val185 → Ala, the dose-response curve was now similar to that observed for the wild type α1a-AR. Panel B, dose-response curve for arachidonic acid production using varying concentrations of (-)-epinephrine. Each data point represents the mean ± S.E. derived from eight separate experiments. Similar to panel A, with the Met292 → Leu mutation there was a shift to the left in the dose-response curve. However, with the double mutation, Met292 → Leu/Val185 → Ala, the dose-response curve was now similar to that observed for the wild type receptor. Panels C and D, maximal IP (C) and arachidonic acid (D) release observed with (-)-epinephrine stimulation of the wild type α1a, and α1b receptors and their corresponding single and double mutants. Data were generated as described in panels A and B and in Fig. 5. The maximum responses for the mutants were not significantly different (using an ANOVA) from that observed with their respective wild type receptors.](http://www.jbc.org/)

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this latter steric constraint can be relieved with the substitution of Val^{185} by alanine.

Recently, mutational analysis of the tachykinin NK-1 receptor revealed that replacement of two residues located at the extracellular surface of the fifth and sixth transmembrane domains (Glu^{193} and Tyr^{272}, respectively) with histidine, enabled Zn^{2+} ions to be coordinated and to act as an antagonist (17). Although the replacement of the wild type residues with histidine may have resulted in altered helical conformations allowing Zn^{2+} coordination, it is of interest that Glu^{193} and Tyr^{272} are exactly one helical turn above Ala^{204} (α_{1b}), and Val^{185} (α_{1a}) of the molecular inducer by perturbing conformation, whereas a histidine in transmembrane VI functions as a repressor, reventing the valine-induced conformational change back to normal. TMV, transmembrane V; TMVI, transmembrane VI.

some other order, as suggested by Pardo et al. (21) and Zhang and Weinstein (22).

At present the only available structural data on receptor activation (23), which is of low resolution, comes from studies of bacteriorhodopsin. These data implicate conformational movement of the sixth and seventh transmembrane segments. However, this receptor is not G-protein-coupled. In support of our notion that the receptor activation involves conformational movement of the third, fifth, and possibly sixth transmembrane domains is the finding that both the third and second intracellular loops, which are contiguous with these transmembrane segments, are involved in G-protein activation. In addition, recent spin labeling data from Khorana and Hubbell (24) indicate that photoexcitation of rhodopsin, a member of the G-protein-coupled receptor family, involves rigid movement of the third helix relative to the others in the ligand binding helical bundle.

The findings of this study also provide potential insights into the evolution of receptor structure. This is evident from the fact that the wild type receptors show little, if any, agonist-independent effector activation, yet mutation of a single residue can result in constitutive activation. This suggests that the native structures have evolved to select residues that repress active state isomerization. Furthermore, if a mutation did occur that introduced such a residue, the resulting constitutively active receptor may have been deleterious to the organism. Propagation of this receptor structure would then only be possible if a simultaneous mutation occurred in the complementary residue in the adjacent transmembrane segment, which could serve as a surrogate “molecular brake” to suppress constitutive activity. Although such a double mutant would have an altered agonist binding profile when evaluated with a variety of synthetic ligands, as observed here with the α_{1a} chimeras, binding of the natural ligands, (−)-epinephrine and (−)-norepinephrine, would be unchanged. This finding, as well as the fact that the α_{1b} and α_{1a}-AR appear to activate all effector pathways similarly, suggests that from the point of view of the organism, such a double mutant would be phenotypically silent. Indeed, based on such considerations, one can postulate that such an evolutionary mechanism may underlie subtype diversity for some classes of G-protein-coupled receptors, such as the α_{1a}-AR. Our ability to discriminate these subtypes may, thus, be due to the development of synthetic ligands that can detect subtle differences between the ligand recognition sites of these subtypes, which can be exploited to develop subtype selective drugs, particularly as different subtypes have been shown to possess different functions, even when expressed in the same tissue. However, further studies will be required, particularly with other classes of G-protein-coupled receptors to confirm or refute this postulate.

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